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A COMPARATIVE STUDY OF THE METABOLISM OF CERTAIN AMINO ACIDS, WITH SPECIAL REFERENCE TO RESPIRATORY EXCHANGE AND HEAT PRODUCTION[1]

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ONE FIGURE

(Received for publication October 10, 1938)

In connection with an investigation of the specific dynamic effects of glycine, alanine and glutamic acid it was necessary to determine the metabolizability of the amino acids, and the factors for computing the respiratory exchange and the heat production. Kriss and Voris ('37) have determined the respiration and energy factors for heart muscle protein, casein and gelatin, and have found them to differ with the type of protein. These differences are presumably related to the amino acid composition of the proteins.

Lusk ('12–'13, '15) calculated the respiration and energy factors for a number of amino acids, including glycine, alanine and glutamic acid, on the basis of theoretical considerations, by accounting for the values of urea formed from them in metabolism. These physiological values (liters of O_2 and CO_2, and calories per gram of nitrogen) are greatly different for the different amino acids. The values for alanine are approximately twice as great as the corresponding values for glycine. The values for glutamic acid are approximately three times as great as those for glycine, while the values for tyrosine are approximately six times the corresponding values for glycine. In general, there is a close inverse correlation between the magnitude of these factors and the nitrogen content of the amino acids. No experimentally determined factors for computing the heat production and the respiratory exchange in the metabolism of amino acids seem to be available.

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[1] Authorized for publication on October 8, 1938, as paper no. 855 in the journal series of the Pennsylvania Agricultural Experiment Station.

The experiments recorded in the present paper are concerned with the determination of the metabolizability of glycine, alanine and glutamic acid, and with the derivation of the respective respiration and energy factors from balances of carbon, nitrogen and energy.

EXPERIMENTAL

The subjects of these experiments were sixteen male albino rats, approaching maturity. The rats weighed somewhat more than 200 gm. each when they were placed on restricted food intake. The first experimental dietary treatment was a basal ration of 8 gm. per day, this ration consisting of 93.7% of calf meal (described by Forbes, Kriss and Miller, '34) and 6.3% of butterfat. During the first few days on this diet the animals lost some weight, but they soon came into equilibrium with the diet, which was adequate for the maintenance of approximate equilibrium of nitrogen and energy. All animals were kept on this diet for a period of approximately 2 weeks before any of the balance experiments were begun.

This preliminary feeding period was followed by a 7-day period on the basal ration during which the feces and urine were collected from each rat.

Following this treatment the animals, divided into three groups, were offered, in addition to the basal ration, supplementary portions of different amino acids[2] in quantities supplying 7500 calories of gross energy per day. Six rats received 2.06 gm. of d-glutamic acid each per day. One animal of this group soon showed hematuria and was therefore discarded. Six other rats received 1.75 gm. of dl-alanine each per day. These two groups received the supplemented diet for a period of 8 days. Feces and urine were collected during the last 5 days. A third group of five rats was offered glycine as a supplement to the basal ration. Only one animal of this group (rat 11) consumed the full amount (2.4 gm. per day) calculated to supply 7500 calories, for a period of 8 days. Feces and urine were also collected from this rat during the last 5 days. The other animals of this group, after receiving the full amount of glycine for a few days, ceased to consume this amount completely. Thereupon the quantity of glycine was reduced to 1.8 gm. (equal to 75% of the original amount) and was fed for 8 days. During the last 5 days of this period feces and urine were collected.

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[2] Purchased from Eastman Kodak Co., Rochester, N. Y.

After the completion of the excreta collection period for the animal (rat 11) which consumed 2.4 gm. of glycine per day, the quantity of glycine fed this rat was also diminished to 1.8 gm. for a period of 8 days, during the last 5 of which excreta were collected.

The urine of each individual rat was analyzed for nitrogen and carbon. Energy was determined in the urine on a composite sample. The feces were composited for each group before being subjected to similar analyses. The basal rations and the amino acids used as supplements were also analyzed for nitrogen, carbon and energy. The excretory products representing the amino acid supplements were determined as the differences between the residues from the basal ration and the supplemented diets.

RESULTS

Table 1 sets forth the analyses of the basal ration and of the amino acid supplements. The analyses indicate a high degree of purity of the amino acids. In table 2 are presented the results of the urinary analysis. These data are characterized by a high degree of consistency. It is of especial interest to note that the carbon: nitrogen ratios representing the urinary constituents of glutamic acid and of glycine are only slightly higher than the carbon: nitrogen ratio (0.428) in urea, while the carbon: nitrogen ratios representing the urinary constituents derived from alanine are more than twice as high as the carbon: nitrogen ratio characteristic of urea. Similarly, the energy: nitrogen ratios representing the urinary constituents of glutamic acid and of glycine are only slightly higher than the energy: nitrogen ratio (5.447 calories per milligram of nitrogen) found in urea, while the energy: nitrogen ratio in the urinary excretions from alanine is more than twice as high as the energy: nitrogen ratio of urea.

These ratios of carbon to nitrogen and of energy to nitrogen indicate that the urinary excretions from the amino acids contain substances other than urea, with higher C: N and energy: nitrogen ratios than those characterizing urea.

TABLE 1

Analysis of basal ration and of amino acid supplements

	%	%	*Calories per gram*
Basal ration	3.27	43.49	4.481
d-glutamic acid	9.30	40.87	3.651
Basal ration	3.27	43.49	4.481
Glycine	18.47	32.25	3.098
Basal ration	3.38	44.05	4.529
dl-alanine	15.50	40.50	4.354

Lusk ('12–'13) made the observation that, in feeding glutamic acid, glycine and alanine to a dog, fractions of unoxidized amino acid were excreted in the urine, these fractions being larger after the ingestion of alanine than after the ingestion of either glycine or glutamic acid. The C: N ratios, as well as the energy: N ratios found in the urine of our rats, are entirely in harmony with these observations of Lusk and may have a similar significance.

In order to arrive at approximate estimates of the quantities of unmetabolized amino acids which passed into the urine, it was first assumed that the differences between the urinary C: N ratios obtained for the particular amino acid and the C: N ratio existing in urea were due entirely to the excretion,

TABLE 2
Urinary analysis

AMINO ACID SUPPLEMENT AND RAT NO.	NITROGEN PER DAY		CARBON PER DAY		ENERGY PER DAY		DIFFERENCES DUE TO AMINO ACIDS				
	Basal ration	Amino acid supplemented ration	Basal ration	Amino acid supplemented ration	Basal ration	Amino acid supplemented ration	Nitrogen	Carbon	Energy	C : N ratio	Energy : N ratio
	mg.	*mg.*	*mg.*	*mg.*	*cals*	*cals.*	*mg.*	*mg.*	*cals.*		
2.06 gm. glutamic acid											
1	167	323	126	197			156	71		0.455	
2	180	337	130	205			157	75		0.478	
3	173	298	126	223			125	97		0.776	
4	174	308	125	200			134	75		0.560	
5	174	320	128	199			146	71		0.486	
Average	174	317	127	205	1381	2254	143	78	873	0.545	6.105
1.8 gm. glycine											
11	173	473	131	280			300	149		0.497	
12	167	469	129	276			302	147		0.487	
13	181	493	136	295			312	159		0.510	
14	154	453	125	284			299	159		0.532	
15	167	451	121	270			284	149		0.525	
Average	168	468	128	281	1425	3235	300	153	1810	0.510	6.033
2.4 gm. glycine											
11	173	589	131	349	1425	4062	416	218	2637	0.524	6.339
1.74 gm. alanine											
21	159	408	117	365			249	248		0.996	
22	160	392	119	371			232	252		1.086	
23	164	386	125	361			222	236		1.063	
24	163	386	121	348			223	227		1.018	
25	153	374	111	370			221	259		1.172	
26	170	384	124	343			214	219		1.023	
Average	162	388	120	360	1303	3903	226	240	2600	1.062	11.504

in the urine, of unmetabolized amino acid; and from these differences the quantities of unoxidized amino acids were computed. Then, in a similar manner, the quantities of unchanged amino acid excreted in the urine were computed on the basis of the differences between the observed urinary energy: nitrogen ratios and the energy: nitrogen ratio of urea. The values obtained for unmetabolized amino acid nitrogen in the urine by the two methods (table 3) are practically identical, and, since they are based on different analytical

TABLE 3

Metabolizability of amino acids

		GLUTAMIC ACID 2.06 GM.	GLYCINE		ALANINE
			2.4 gm.	1.8 gm.	1.74 gm
Income					
Energy, calories		7.521	7.435	5.576	7.576
Carbon, grams		0.842	0.774	0.581	0.705
Nitrogen, grams		0.192	0.443	0.332	0.270
Outgo					
Feces					
Energy, calories		0.569	......	......	......
Carbon, grams		0.056	......	......	......
Nitrogen, grams		0.010	......	......	......
Urine					
Energy, calories		0.873	2.637	1.810	2.600
Carbon, grams		0.078	0.218	0.153	0.240
Total nitrogen, grams		0.143	0.416	0.300	0.226
Unmetabolized amino acid N	grams[1]	0.004	0.031	0.019	0.061
	grams[2]	0.003	0.033	0.016	0.062
Metabolized amino acid N	grams[1]	0.139	0.385	0.281	0.159
	grams[2]	0.138	0.383	0.284	0.164
	per cent[1]	97.20	92.55	93.67	70.35
	per cent[2]	96.50	92.07	94.67	72.57
Metabolizable					
Energy, calories		6.079	4.798	3.766	4.976
Carbon, grams		0.708	0.556	0.428	0.465
Energy, per cent		80.83	64.53	67.54	65.68
Carbon, per cent		84.09	71.83	73.67	65.96

[1] Calculated on the basis of the C: N ratios.

[2] Calculated on the basis of calories: N ratios.

procedures, their agreement can be considered as serving to substantiate the essential correctness of the assumption involved.

It should be stated that the foregoing calculations do not preclude the possibility of the presence in the urine, besides urea and unoxidized amino acid, of other products of incomplete oxidation of the amino acid, with a combined C: N ratio and a combined energy: nitrogen ratio corresponding to the ratios found in the unchanged amino acid. This and any other theoretical considerations, however, which might affect to some extent the partitioning of the urinary nitrogen, would be without significant effect on the final respiration and energy values on which the graphs in figure 1 are based, inasmuch as those values are expressed in relation to the total urinary nitrogen.

The quantities of nitrogen in the urine representing the amino acids metabolized were calculated as the differences between the total urinary nitrogen and the unmetabolized amino acid nitrogen. Calculated from the C: N ratios, the average quantities of nitrogen in the urine derived from the amino acids metabolized constitute 97.20% of the total urinary nitrogen for glutamic acid, 92.55% and 93.67%, respectively, for the higher and lower levels of glycine, and 70.35% for alanine. The same values calculated from the calories: N ratios are 96.50% for the glutamic acid, 92.07% and 94.67%, for the two levels of glycine, and 72.57% for alanine. These values are in substantial agreement with those found by Lusk ('12–'13), in the dog, on the basis of determinations of urea plus ammonia nitrogen, and total nitrogen, in the urine. Lusk's estimate for the deaminized portion of alanine was 70%, and for glycine 96.06%, and 94.46%, in two experiments.

In all of the foregoing calculations it was tacitly assumed that the protein metabolism was not altered by the addition of the amino acid supplements to the basal ration. Direct proof of the correctness of this assumption is not feasible, but it is substantiated indirectly by the consistent character of the results. An alternative assumption, that the contribution to the protein metabolism by the basal ration in the supermaintenance periods is represented by the difference between the total urinary nitrogen of the supermaintenance period and the total amino acid nitrogen ingested or absorbed, leads to the calculation of C: N ratios in the urinary residues from the amino acids, which are in all cases considerably higher than the C: N ratios found in the urine from the basal ration. This would not be consistent with the large quantities of urea known to be formed from the amino acids and excreted in the urine, especially in the case of glycine feeding. The second assumption must, therefore, be discarded in favor of the first.

The results of the fecal analyses were such as to indicate that glycine and alanine were completely absorbed from the intestinal tract, and that small quantities of glutamic acid were eliminated in the feces. The differences between the daily fecal outgo from the ration supplemented with glutamic acid and the basal ration were 10 mg. nitrogen, 56 mg. carbon, and 569 calories. These values were all positive and their relations to each other are not much different from those existing in the amino acid. With glycine and alanine the differences representing fecal elimination were negligibly small. It is possible that elimination of small quantities of glutamic acid through the intestine occurred because of a relatively slow rate of absorption of this amino acid (Wilson and Lewis, '29).

In table 3 are presented the balances of nitrogen, carbon and energy representing the different amino acids. The metabolizable energy or carbon of the amino acids is computed as the difference between the total energy or carbon of the substance ingested and the corresponding energy or carbon, of the excreta. In this computation the energy of the urine was not corrected to represent nitrogen equilibrium.

Of the total nitrogen ingested as amino acids the following percentages were eliminated in the urine: from glutamic acid, 74.48%, from glycine, 93.91% and 90.36%, for the higher and lower levels, respectively, and from alanine, 83.70%.

Of the energy ingested as glutamic acid 80.83% was metabolized; the energy of glycine was metabolized to the extent of

TABLE 4

Determination of factors for computing the respiratory exchange and the heat production in the metabolism of glutamic acid, glycine and alanine

	GLUTAMIC ACID 2.06 GM.	GLYCINE		ALANINE 1.74 GM.
		2.4 gm.	1.8 gm.	
Intake, computed per 100 gm. of pure amino acid				
Energy, calories	365.10	309.80	309.80	435.40
Nitrogen, grams	9.52	18.66	18.66	15.73
Carbon, grams	40.79	31.98	31.98	40.42
Hydrogen, grams	6.17	6.72	6.72	7.92
Oxygen, grams	43.51	42.64	42.64	35.93
Outgo				
Feces				
Energy, calories	28.21			
Nitrogen, grams	0.50			
Carbon, grams	2.78			
Hydrogen, grams	0.32			
Oxygen, grams	2.29			
Urine				
Energy, calories	55.07	118.28	112.58	180.96
Total nitrogen, grams	9.02	18.66	18.66	15.73
Unmetabolized amino acid N, grams	0.25	1.44	1.12	4.52
Metabolized (urea) N, grams	8.77	17.22	17.54	11.21
Carbon, grams	4.92	9.78	9.52	16.70
Hydrogen, grams	1.41	2.98	2.91	3.88
Oxygen, grams	6.15	13.13	12.58	16.74
Metabolized				
Energy, calories	281.82	191.52	197.22	254.44
Carbon, grams	33.09	22.20	22.46	23.72
Hydrogen, grams	4.44	3.74	3.81	4.06
Oxygen, grams	35.07	29.51	30.06	19.19
Intramolecular H_2O				
Hydrogen, grams	4.38	3.69	3.76	2.40
Oxygen, grams	35.07	29.51	30.06	19.19
Carbon oxidized to CO_2, grams	33.09	22.20	22.46	23.72
Hydrogen oxidized to H_2O, grams	0.06	0.05	0.05	1.66
CO_2 produced, grams	121.33	81.40	82.35	86.97
CO_2 produced, liters	61.81	41.47	41.95	44.30
O_2 required to oxidize C, grams	88.24	59.20	59.89	63.25
O_2 required to oxidize H, grams	0.48	0.40	0.40	13.28
Total O_2 required, grams	88.72	59.60	60.29	76.53
Total O_2 required, liters	62.09	41.71	42.19	53.56
Respiratory quotient	0.995	0.994	0.994	0.827
Liters O_2 required per gram urinary N	6.88	2.24	2.26	3.40
Liters CO_2 produced	6.85	2.22	2.25	2.82
Calories metabolized per gram urinary N	31.24	10.26	10.57	16.18
Calories per liter O_2	4.491	4.592	4.675	4.751
Liters O_2 required per gram N of amino acid metabolized	7.08	2.42	2.41	4.78
Liters CO_2 produced per gram N of amino acid metabolized	7.05	2.41	2.39	3.95
Calories metabolized per gram N of amino acid metabolized	32.13	11.12	11.24	22.70

64.53% (for the higher level) and 67.54% (for the lower level). The metabolizable energy of alanine was 65.68% of the total.

The percentages of carbon metabolized were 84.09 for glutamic acid, 71.83 and 73.67 for glycine, and 65.96 for alanine.

The relatively low metabolizability of the energy of glycine as compared with glutamic acid is apparently due to the greater quantity of urea formed from the former, while the relatively low metabolizability of the energy of alanine is apparently due, to some extent, to the relatively large quantity of unchanged amino acid eliminated in the urine.

The derivation of the respiration and energy factors for the different amino acids is shown in table 3. The computations are on the basis of pure amino acids.

The values for nitrogen, carbon and energy of feces and urine, per 100 gm. of amino acid, were computed on the basis of the data given in table 2, in relation to the nitrogen content of the amino acids. The values for hydrogen and oxygen in the feces from glutamic acid were computed from the nitrogen on the assumption that the fecal excretion was in the form of the amino acid. The values for hydrogen and oxygen in the urine were computed on the basis of the calculated values of urea and of unmetabolized amino acid present. All other calculations for the derivation of the respiration and energy factors are self-explanatory.

The final results obtained with each of the amino acids tested (the values for glycine being given as the average for the two levels) are summarized as follows:

	Glutamic acid	*Glycine*	*Alanine*
Respiratory quotient	0.995	0.994	0.827
Calories metabolized per liters O_2	4.491	4.634	4.751
Liters O_2 required per gram urinary N	6.88	2.25	3.40
Liters CO_2 produced per gram urinary N	6.85	2.24	2.82
Calories metabolized per gram urinary N	31.24	10.42	16.18
Liters O_2 required per gram N of amino acid metabolized	7.08	2.42	4.78
Liters CO_2 produced per gram N of amino acid metabolized	7.05	2.40	3.95
Calories metabolized per gram N of amino acid metabolized	32.13	11.18	22.70

The values for O_2, CO_2 and calories per gram of total urinary nitrogen do not differ very much from the corresponding values expressed per gram of nitrogen of amino acid metabolized, in the case of glutamic acid or of glycine. In the case of alanine, however, the factors expressed per gram of nitrogen of the amino acid metabolized are considerably higher than those expressed per gram of total urinary nitrogen. This is accounted for by the fact, previously shown (table 3), that relatively small quantities of unmetabolized glutamic acid or glycine appeared in the urine, while a considerable proportion of the ingested alanine was eliminated in the urine in unchanged form. The respiration and energy factors for all three amino acids under consideration, expressed per gram of nitrogen of amino acid metabolized, agree very closely with the corresponding theoretical values calculated by Lusk ('12–'13, '15). The factors per gram of total urinary nitrogen, however, correctly apply to the experimental conditions.

A close examination of the respiration and energy factors which have been determined for the different amino acids, in the present study, and of the factors for proteins reported previously by Kriss and Voris ('37), in relation to the elementary composition of these substances, reveals that while there is, in general, a fairly close correlation between these factors and the nitrogen content of the substances, a still closer correlation exists between these factors and the ratios of carbon to nitrogen in the materials. The latter relationship is shown graphically in figure 1.

The graphs show consistently higher values for O_2, CO_2 and calories per gram of total urinary nitrogen, with the proteins than with the single amino acids, thus indicating that the metabolism (oxidation) was more complete with the former than with the latter. The graphs representing the amino acids do not and probably would not apply to sulphur-containing amino acids.

While the data on which figure 1 is based are few, they clearly indicate that when more experimental data of this sort become available it will probably be possible to derive

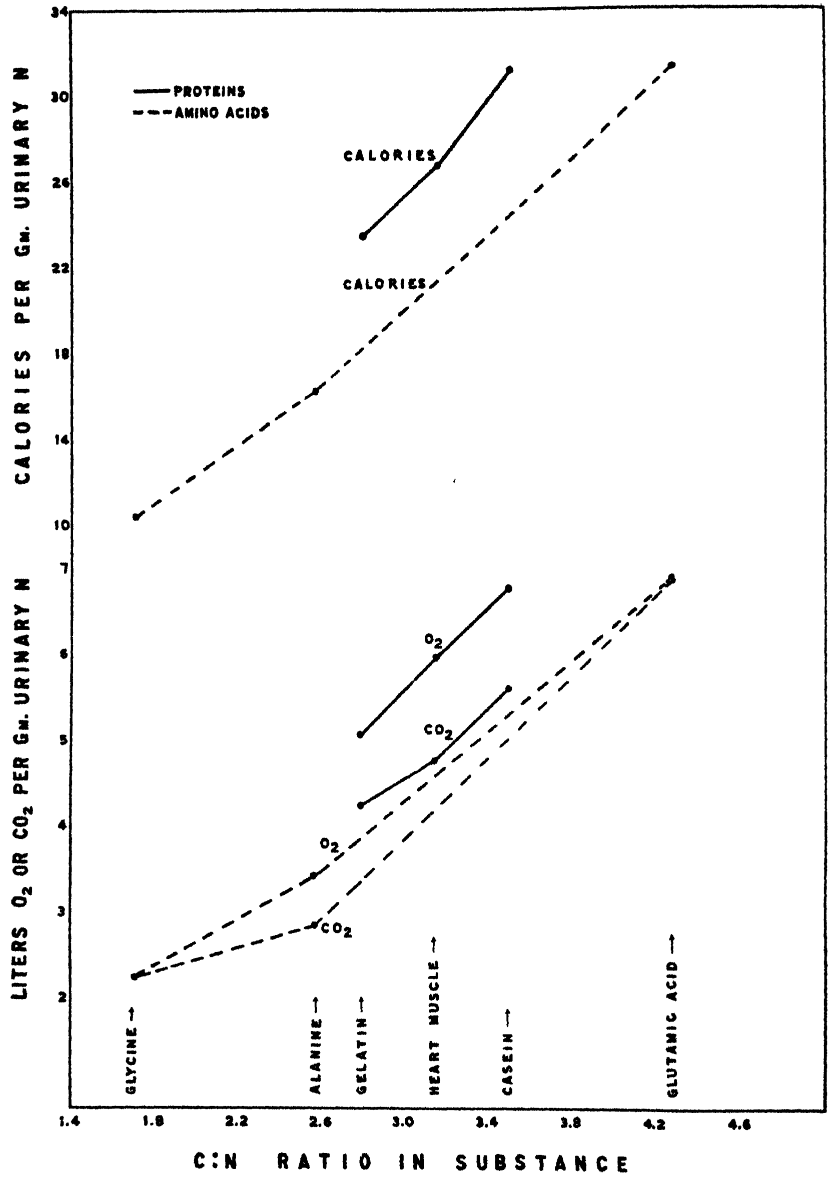

Fig. 1 Relation of respiration and energy factors for amino acids and proteins to the ratios of carbon to nitrogen in these substances.

a general formula, for practical use in indirect calorimetry, for the calculation of respiration and energy factors for any protein, and possibly for any amino acid. Tentatively, there seems to be good reason to suggest that the factors for proteins the C : N ratios of which lie within the range of 2.79 (gelatin) and 3.50 (casein) can be calculated from the C : N ratios in the substance, on the basis of the data for proteins represented in figure 1, by interpolation.

SUMMARY

Glutamic acid fed to rats as a supplement to a mixed maintenance ration, in a quantity supplying 7.5 kilogram-calories of gross energy per day, was absorbed to the extent of 95%, while glycine fed as a supplement to the same basal ration, in quantities supplying 7.4 and 5.6 kilogram-calories, and alanine fed in a quantity supplying 7.5 kilogram-calories per day, were completely absorbed. From the ratios of carbon to nitrogen and of energy to nitrogen in the urine, it was calculated that approximately 3% of glutamic acid, 7% of glycine and 29% of alanine, respectively, were excreted in the urine in the form of the unoxidized amino acids, the remaining portions of the urinary nitrogen, derived from the amino acids, representing the amino acids metabolized. The metabolizable energy of glutamic acid was found to be 80.8% of its gross energy, while the metabolizability of the energy of alanine was only 65.68%, and that of glycine 64.53%, at the higher level, and 67.54% at the lower level of feeding.

Factors were determined for computing the respiratory exchange and the heat production in the metabolism of each of the three amino acids. These factors were found to be closely correlated with the ratios of carbon to nitrogen in the different amino acids. A similar correlation was found to exist between the respiration and energy factors for some proteins, namely, casein, gelatin and heart muscle protein, and the ratios of carbon to nitrogen in these substances. A graphic representation of these relationships indicates that the proteins were more completely metabolized than were the

single amino acids. A tentative method has been suggested for making practical use of these relationships in indirect calorimetry.

The author is indebted to Mr. Virgil V. Herring for carrying out the determinations of carbon in connection with this investigation.

LITERATURE CITED

FORBES, E. B., M. KRISS AND R. C. MILLER 1934 The energy metabolism of the albino rat in relation to the plane of nutrition. J. Nutrition, vol. 8, p. 535.

KRISS, MAX, AND LEROY VORIS 1937 A further contribution to the derivation of factors for computing the gaseous exchange and the heat production in the metabolism of protein. J. Nutrition, vol. 14, p. 215.

LUSK, G. 1912–1913 Animal calorimetry. Fifth paper. The influence of the ingestion of amino-acids upon metabolism. J. Biol. Chem., vol. 13, p. 155.

——— 1915 Animal calorimetry. Eleventh paper. An investigation into the causes of the specific dynamic action of the foodstuffs. J. Biol. Chem., vol. 20, p. 555.

WILSON, R. H., AND H. B. LEWIS 1929 Comparative studies of the metabolism of amino acids. II. The rate of absorption of amino acids from the gastro-intestinal tract of the white rat. J. Biol. Chem., vol. 84, p. 511.

THE LEVEL OF INORGANIC PHOSPHORUS IN THE BLOOD OF DAIRY CATTLE [1]

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(Received for publication October 1, 1938)

In connection with a study of phosphorus deficiency in the cattle of Pennsylvania it was necessary to establish the normal content of inorganic phosphorus in the whole blood. A series of determinations of this value were made, therefore, with well-fed cattle as the experimental subjects.

As pointed out by Hamersma ('37), diverse results have been obtained from determinations of inorganic blood phosphorus in different laboratories in accord with the method employed—the estimations having usually been made on the serum or the plasma.

Under field conditions such that the blood cannot be chilled or analyzed at once, it is more desirable to use whole blood for the determination, for reasons discussed by Stare and Elvehjem ('32). These authors, and Van Landingham, Henderson and Bowling ('35), have determined the inorganic phosphorus of the whole blood of immature dairy cattle, and both these groups of workers used the method of Fiske and Subbarow.

Recently Van Landingham and co-workers ('38) presented values for the inorganic phosphorus of the blood of successive periods of lactation, as follows:

1st lactation 4.95 mg. per 100 ml.
2nd lactation 4.81 mg. per 100 ml.
3rd lactation 4.35 mg. per 100 ml.
4th lactation 4.33 mg. per 100 ml.

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[1] Authorized for publication on September 9, 1938, as paper no. 850 in the journal series of the Pennsylvania Agricultural Experiment Station.

In addition to the recognized effects of age and of feed on the inorganic phosphorus level in blood, the stage of lactation (Van Landingham, Henderson and Bowling, '38), and the amount of milk produced (Van Landingham, Henderson and Bowling, '38; Becker, Neal and Shealy, '33; Eckles, Gullickson and Palmer, '32; Groenewald, '35) are said also to exert appreciable effects.

Becker, Neal and Shealy ('33) give values for nursing cows of 2.87 mg., for dry cows of 3.80 mg. and for steers and heifers of 4.91 mg. per 100 cc., all representing cattle in phosphorus deficient areas.

The specific purposes of the present study were to establish standard, normal values for the inorganic phosphorus of the whole blood of dairy cattle, and to study the variations in these values resulting from differences in conditions as to breed, age, milk production, reproductive status and other factors on which information could be obtained.

EXPERIMENTAL

Inorganic phosphorus was determined on blood samples from over 200 cows and heifers by the method of Youngburg and Youngburg ('30). Immediately after drawing the sample, the quantity of 2 cc. was pipetted into 8 cc. of 10% trichloracetic acid. The samples were filtered as soon as possible, being kept cold in the meantime, though preliminary studies indicated that these precautions were probably unnecessary.

The animals of two separate herds at the college, one of which is maintained primarily for instructional and the other for research purposes, were used. No attempt was made to include every animal, and, in any case in which the drawing of the blood caused excitement of the animal, the sample was rejected.

The research herd, referred to as herd 1, was receiving a standard, balanced, concentrate mixture, and timothy hay, and either corn or alfalfa silage. The instructional herd, designated herd 2, received a balanced ration of oats, corn, peanut bran, soy bean meal, brewers grains, wheat bran, molasses and salt, and mixed or alfalfa hay and corn silage.

All samples were taken during the spring, when the animals had been away from pasture for 4 months or longer.

The data were grouped in several different ways. In some cases, in which significant differences between the average values for groups which were studied were not found, it is possible that a study of larger populations might uncover definite differences.

In herd 1, the values for the animals on corn silage and for those on alfalfa silage were not significantly different; neither were there important differences in accord with the breed and the age of adult cattle, though in the case of heifers the age groups did differ as to blood phosphorus, and significant differences were found between the milking and the dry cows. Two cows that were being dried off, and were giving less than 10 pounds of milk daily, were included in the dry-cow group. All other cows were giving 25 pounds or more of milk daily. Within the small group of twenty-six milking cows no definite correlations between milk production and inorganic phosphorus in the blood was found.

In herd 2, only five of the cows tested were dry, and their inorganic blood phosphorus values were similar to the average for those which were giving milk. There was no difference between the averages for cows giving 10 to 24 and those giving 25 to 40 pounds of milk, and a small difference between those giving 25 to 40 pounds and others giving over 40 pounds of milk daily was not statistically significant. In this herd, also, no differences were found between breeds nor between the 2- to 3-year-old and the 4- to 5-year-old milking cows, but a significant difference was found between averages for 4- to 6-year-old cows and cows which were more than 6 years old.

In both herds cows which had calved within 2 weeks were excluded, since their inorganic blood phosphorus values were quite variable, and in most cases were abnormally high. Only a few males were examined. They were all young, and the values obtained could not be distinguished from those for heifers of the same age.

The averages for the groups of heifers from the two herds were almost identical. They were therefore combined, and were then divided into groups differing as to age by approximately 6 months. The relation of age to level of inorganic phosphorus in the blood of immature cattle is clearly shown in tables 1 and 2. The range of individual values also descended uniformally with advance in age, except for the low value of 4.1 mg. per 100 ml. in the 14- to 19-month-old group. The next lowest value in this group was 4.8 mg., which is in line with the lower limit of the other age groups. If the average values of the five age groups are plotted against the ages of these groups the result is a nearly straight line representing diminishing inorganic blood phosphorus with increasing age. In herd 2 there was also a significant difference between the inorganic blood phosphorus of cows 4 to 6 years of age, and cows 6 years of age or older.

The average values for inorganic blood phosphorus of the milking cows in herd 2, and of the dry cows in both herds were virtually the same, 4.33 mg. and 4.35 mg., respectively, per 100 ml. of whole blood. The value 4.11 mg. for the milking cows of herd 1 is significantly lower than the above values, while the heifers and dry cows of this herd gave values in harmony with those of herd 2. This inconclusive evidence indicates that the ration for the cows in herd 1 was not sufficiently high in phosphorus to enable cows in full lactation to maintain a normal level of blood phosphorus.

The results suggest that milk production has little or no influence on the level of inorganic phosphorus in the blood when the animals are under optimum conditions of feed and management. This does not preclude the probability that milk production has an effect on the blood phosphorus values of animals on restricted feed intake, or on rations of low phosphorus content.

In field studies carried out in connection with this work, very few low values for the inorganic blood phosphorus of dry cows were found, even with low phosphorus intake. A similar observation was also made by Becker and associates

TABLE 1

Average inorganic phosphorus contents of the whole blood of dairy cattle. Milligrams per 100 ml.

GROUP	DESCRIPTION	NUMBER	AGE	DAILY MILK	MEAN, INORGANIC P	PROBABLE ERROR OF MEAN	STANDARD DEVIATION	RANGE, INORGANIC P
	Herd 1							
			months	*pounds*				
1	Milking cows	26	57	44	4.11	0.06	0.48	3.3–5.3
2	Dry cows	12	76	..	4.39	0.07	0.36	3.7–4.9
	Herd 2							
3	Cows in milk, 2 to 3 years old	25	40	30	4.42	0.05	0.38	3.7–5.3
4	Cows in milk, 4 to 5 years old	22	60	35	4.45	0.04	0.31	3.9–5.1
5	Cows in milk, 6 years and over	17	97	30	4.11	0.07	0.40	3.2–5.0
6	Cows giving 10 to 24 pounds milk	20	71	18	4.40	0.06	0.42	3.7–5.3
7	Cows giving 25 to 40 pounds milk	30	54	31	4.37	0.04	0.36	3.7–5.1
8	Cows giving over 40 pounds milk	14	66	54	4.21	0.07	0.39	3.2–4.8
9	All milking cows of this herd	64	62	32	4.33	0.04	0.49	3.2–5.3
	Herds 1 and 2 combined							
10	Calves 1 to 7 months old	26	4.2	..	6.42	0.06	0.53	5.4–7.4
11	Calves 8 to 13 months old	16	9.8	..	6.01	0.10	0.58	5.2–7.1
12	Calves 14 to 19 months old	20	16.1	..	5.36	0.08	0.52	4.1–6.0
13	Calves 20 to 24 months old	16	22.1	..	4.94	0.05	0.31	4.4–5.6
14	Calves 25 to 30 months old	23	27.0	..	4.70	0.06	0.42	4.0–5.6
15	All milking cows	90	60	33	4.28	0.03	0.42	3.2–5.3
16	All dry cows	17	77	..	4.35	0.06	0.36	3.6–4.9
17	All cows	111	63	..	4.29	0.03	0.42	3.2–5.3

('33); and other workers (Van Landingham, Henderson and Bowling, '35; Eckles, Gullickson and Palmer, '32; Groenewald, '35) conclude that lactation lowers the level of inorganic phosphorus in the blood.

As a normal value for inorganic phosphorus in the blood of milking cows the average for such cows in herd 2 is considered reliable. This value, 4.33 mg. ± 0.04 mg., with a standard deviation of 0.59 mg. per 100 ml., represents sixty-four animals, among which were representatives of all the common dairy breeds. These cows were giving from 10 to 72 pounds of milk daily. The value for dry cows is similar.

TABLE 2

The significance of the differences in the inorganic phosphorus contents of the whole blood of groups of dairy cattle[1]

GROUP	DESCRIPTION	MEAN DIFFERENCE	P. E. OF DIFFERENCE	ODDS OF SIGNIFICANCE
		Milligrams per 100 ml.		
2 vs. 1	Dry cows vs. milking cows, herd 1	0.279	0.094	22: 1
9 vs. 1	Milking cows, herd 2 vs. herd 1	0.227	0.075	23: 1
4 vs. 5	Middle aged vs. old cows, herd 2	0.339	0.079	260: 1
10 vs. 11	1- to 7- vs. 8- to 13-month old calves	0.402	0.121	39: 1
11 vs. 12	8- to 13- vs. 14- to 19-month-old calves	0.653	0.125	2300: 1
12 vs. 13	14- to 19- vs. 20- to 24-month-old calves	0.416	0.093	395: 1
13 vs. 14	20- to 24- vs. 25- to 30-month-old calves	0.248	0.078	28: 1

[1] The first of the two groups in each comparison has the higher value.

The values for the age groups of calves (mostly heifers), as given in table 1, represent 101 animals and should be fairly reliable for immature cattle under winter conditions.

SUMMARY

Inorganic phosphorus was determined in the whole blood of over 200 cattle—mostly cows and heifers—in normal states of health and nutrition.

The inorganic phosphorus of the blood of immature cattle diminished with increase in age in an approximately regular manner.

As to the effect of age on inorganic blood phosphorus values of milking cows, no such effect was observed as between cows 2 to 3 years old compared with cows 4 to 5 years old, but aged cows had significantly less inorganic phosphorus in their blood than did middle aged cows.

The effects of lactation on inorganic blood phosphorus were questionable.

No influences of the breed of cow or of the kind of roughage consumed on inorganic blood phosphorus values, were observed.

The average inorganic phosphorus content of the blood of sixty-four milking cows was 4.33 mg. ± 0.04 mg., with a standard deviation of 0.59 mg. per 100 ml.

The corresponding value for dry cows was approximately the same.

ACKNOWLEDGMENT

My thanks are due to Drs. S. I. Bechdel and J. F. Shigley for the drawing of the blood samples, and to Dr. E. B. Forbes for helpful criticism throughout.

LITERATURE CITED

BECKER, R. B., W. M. NEAL AND A. L. SHEALY 1933 Stiffs or sweeny (phosphorus deficiency) in cattle. Fla. Agr. Exp. Sta. Bul. 264, 27 pp.

ECKLES, C. H., T. W. GULLICKSON AND L. S. PALMER 1932 Phosphorus deficiency in the rations of cattle. Minn. Agr. Exp. Sta. Tech. Bul. 91, 118 pp.

GROENEWALD, J. W. 1935 The influence of rations low in certain minerals on the composition of the blood and milk of cows and on the blood of their progeny. Onderstepoort J. Vet. Sci. and Animal Industry, vol. 4, pp. 93–165.

HAMERSMA, P. J. 1937 Chemical blood studies VII. Onderstepoort J. of Vet. Sci. and Animal Industry, vol. 8, pp. 441–483.

STARE, F. J., AND C. A. ELVEHJEM 1932 The phosphorus partition in the blood of rachitic and non-rachitic calves. J. Biol. Chem., vol. 97, pp. 511–524.

VAN LANDINGHAM, A. H., H. O. HENDERSON AND G. A. BOWLING 1935 Chemical composition of the blood of dairy cattle. I. The effect of age and phosphorus intake on the calcium and inorganic phosphorus content of whole blood of dairy heifers. J. Dairy Sci., vol. 18, pp. 557–572.

——— 1938 The normal concentration of inorganic phosphorus in the blood of lactating dairy cows and factors affecting it. J. Dairy Sci., vol. 21, abstracts, pp. 112–113.

YOUNGBURG, GUY E., AND MAMIE V. YOUNGBURG 1930 Phosphorus metabolism. 1. A system of blood phosphorus analysis. J. Lab. and Clin. Med., vol. 26, pp. 158–166.

THE FACTOR I (VITAMIN B_6) REQUIREMENT OF THE RAT

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THREE FIGURES

(Received for publication September 19, 1938)

The vitamin B complex has now been separated into five distinct components: thiamin, riboflavin, nicotinic acid amide, factor I (vitamin B_6), and factor II (filtrate factor). Of these, thiamin (Williams and Cline, '36), riboflavin (Euler, et al., '35), and nicotinic acid amide (Elvehjem, et al., '37) have been available in crystalline form for some time. With the availability of factor I in crystalline form (Lepkovsky, '38), it became possible for the first time to determine the rat's requirement for this vitamin. Accordingly five groups of rats were fed various levels of crystalline factor I. The levels chosen were 50, 20, 10, 3 and 1 micrograms daily, and each group included a minimum of five animals.

EXPERIMENTAL

Twenty-one-day-old female rats of the Long-Evans strain were placed in cages with raised screened bottoms to minimize the effect of coprophagy. The basal diet consisted of sucrose 59, extracted casein [1] 27, fat (primex) 8, cod liver oil 2, and salt mixture no. 185 (McCollum and Simmonds, '18) 4. Water bottles attached to the cages provided fresh water at all times. All rats were observed daily and weighed at weekly intervals.

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[1] The casein was extracted with 20% iso-propanol. Repeated extractions were made until the washings from the casein were entirely free of color. This required ten individual extractions. After the completion of the iso-propanol extractions, the casein is given two washings with acetone; and then dried.

For the first 2 weeks immediately after weaning, the rats were fed the basal diet but no supplements. At the end of the period, the rats were transferred to individual wire-screen floored cages, and the basal diet was supplemented with 25 micrograms each of thiamin and riboflavin daily. These supplements were measured out into Syracuse watch glasses and placed in the cage. The rats became rapidly conditioned to this feeding procedure and avidly consumed the dose as soon as it was placed in the cage. The above dietary regime was maintained for 2 weeks, at the end of which period factor II (daily dose 0.5 cc.) was supplied as a beef liver extract prepared according to the method described by Lepkovsky, Jukes and Krause ('36). When growth ceased and dermatitis appeared, crystalline factor I was fed. Crystalline factor I was prepared from rice bran extract by the method of Lepkovsky ('38).[2]

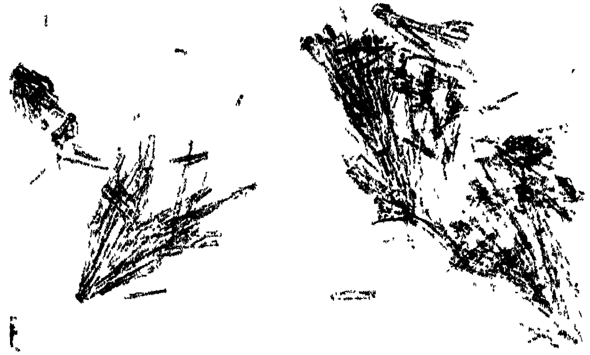

Fig. 1 Crystalline factor I. × 290.

The factor I crystallized as needles, largely in the form of rosettes. They formed on the stirring rod, and on the sides and bottom of the beaker. A small amount of gummy material had settled on the bottom of the beaker. The crystals were loosened, and by careful stirring were suspended in the alcohol-acetone mixture and poured out quickly to remove a minimum of the gummy material. The dry crystals so obtained had a slight yellowish tinge. The gummy layer in the bottom of the beaker and the crystals remaining behind were dissolved in a minimum of water and again precipitated with alcohol and acetone, and another smaller crop of crystals was obtained. About 140 mg. of crystalline factor I were thus obtained from about 1 liter of the crude eluate. Figure 1 shows the form of the crystals.

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[2] We wish to thank Doctor Lepkovsky for making available to us the manuscript describing the method for preparing factor I while it was still in press.

Adequacy of factor II was checked on chickens and on rats, the method of Jukes ('37) being used for the former and the procedure of Lepkovsky, Jukes and Krause ('36) being followed for the latter. That the liver extract was free of factor I is evidenced by the cessation of growth and the appearance of dermatitis in rats fed the basal diet supplemented with all the known water soluble vitamins exclusive of factor I.

Results obtained with different levels of factor I

Figure 2 shows the growth secured with different levels of factor I. At 50, 20 and 10 microgram levels there was an immediate resumption of growth and a marked improvement in the dermatitis. Even in cases of the most florid type of dermatitis, involving the ears, paws and face, complete healing occurred within 10 days so that the rats were indistinguishable from normal animals.

At the 1 microgram level, very little growth was made and healing of the dermatitis was very slow. On the 3 microgram level, the healing was much more marked and in some cases almost complete. While 1 and 3 micrograms of factor I were insufficient to bring about a complete cure, some growth took place, and the life of the rats was greatly prolonged.

The survival period of rats deprived of factor I rarely extends beyond 50 to 60 days.

The effect of inanition in factor I deficiency

Since the water-soluble vitamins tend to affect appetite, it seemed of interest to compare the growth responses of animals fed the factor I-free diet and allowed all the food desired, with the growth responses of rats fed the basal diet including factor I but kept on a restricted food intake.

Forty rats consisting of twenty pairs of litter mates were divided into two groups at the time of weaning. One group was placed on the factor I-deficient regime. The food for each animal of this group was weighed separately each day. Each animal of the second group was fed the average daily ad libitum food intake of the first group plus 20 micrograms of factor I. A third group of rats receiving 20 micrograms of factor I and food ad libitum was included as a positive control group. Figure 3 shows the average growth of these three groups of animals.

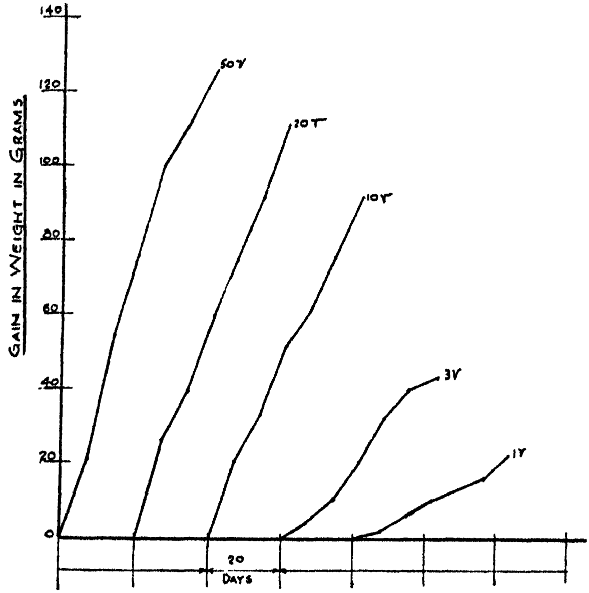

Figure 2 shows the gain in weight in grams of groups of rats fed various levels of crystalline factor I. The levels are 50, 20, 10, 3 and 1 micrograms. Each group includes a minimum of five rats.

The animals on the restricted food intake and receiving twenty micrograms of factor I showed a voracious appetite, finishing the allotted ration within a few minutes after it was placed in the cage.

The average amount of food consumed daily by the groups of deficient animals varied from 4.5 to 4.9 gm. The severity of the dermatitis seemed to have no correlation with the food intake. With the same average food intake in the two groups, 20 micrograms daily of factor I resulted in a heavier body

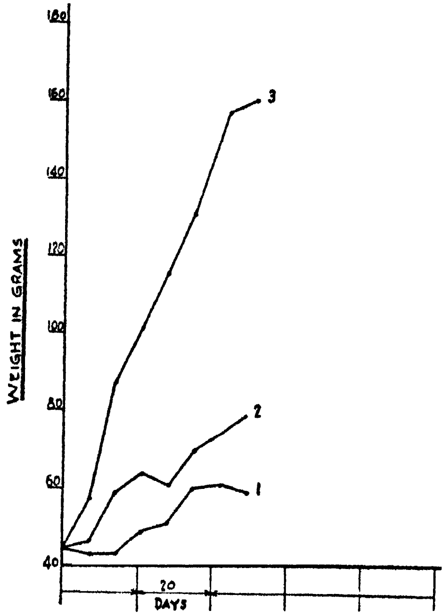

Figure 3 shows the results obtained with three groups of rats as follows: factor I-deficient group, a limited food control group, and a normal control group. Curve 1 shows typical depletion curve for factor I. These rats received the basic diet unsupplemented for 2 weeks; 25 micrograms of thiamin and 25 micrograms of riboflavin were then added, and at the end of an additional 2 weeks 0.5 cc. liver filtrate was added. Curve 2 shows the growth obtained with the same supplements as shown in curve 1 plus 20 micrograms of factor I except with the food limited to the average amount consumed by the factor I-deficient rats. Curve 3 shows the growth obtained when all the vitamin B complex factors are supplied as follows: 25 micrograms of thiamin, 25 micrograms of riboflavin, 20 micrograms crystalline factor I, and 0.5 cc. liver filtrate. These rats received an unlimited food supply.

weight in the limited group. In several cases individual factor I-depleted rats had a larger food intake than some of the animals maintained on the restricted food intake plus factor I,[3] but in spite of this difference weighed less than the latter. Aside from stunting, outwardly the rats on the limited food intake were indistinguishable from normal animals.

The three groups of rats were sacrificed on the seventieth day of life or 49 days after the initiation of the experiment. One outstanding finding was the complete atrophy of the thymus in the factor I deficient group. In the restricted food intake group these glands were present though reduced in size. The ovaries showed no development in either group. The lack of ovarian development was consistent with the observation that in no case, was the vaginal introitus established at the time the experiment was terminated.

Another interesting finding was the presence of considerable amounts of fat in the animals maintained on the restricted food intake. The factor I-deprived rats, on the other hand, showed a complete absence of fat storage. This finding is additional evidence that factor I makes for a greater or more efficient food utilization.

SUMMARY

1. There is slight but recognizable response of rats on a factor I-deficient diet to as little as 1 microgram of crystalline factor I.

2. Ten micrograms of factor I nearly satisfied the rat's requirements for this vitamin.

3. Rats receiving factor I but limited in their food intake to that of rats on the factor I-deficient diet appeared to utilize food more efficiently. This was evidenced by a greater body weight and the presence of fat in their tissues.

4. In the rats on the factor-I deficient diet, the thymus atrophies more quickly than in those receiving the same amount of food with factor I.

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[3] It should be recalled that rats receiving factor I were fed the average daily food intake of the entire group of deficient animals.

LITERATURE CITED

ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG AND D. W. WOOLLEY 1937 Relation of nicotinic acid and nicotinic acid amide to the canine blacktongue. J. Am. Chem. Soc., vol. 59, p. 1767.

EULER, H. V., P. KARRER, M. MALMBERG, K. SCHÖPP, F. BENZ, B. BECKER AND P. FREI 1935 Synthese des lactoflavins (vitamins B_2) und andere Flavine. Helv. Chim. Acta., Bd. 18, S. 522.

JUKES, T. 1937 Further observations on the assay, distribution and properties of the filtrate factor. J. Biol. Chem., vol. 117, p. 11.

LEPKOVSKY, S. 1938 The isolation of factor I in crystalline form. J. Biol. Chem., vol. 124, p. 125.

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, p. 557.

MCCOLLUM, E. V., AND N. SIMMONDS 1918 A study of the dietary essential, water soluble B_1 in relation to its solubility and stability toward reagents. J. Biol. Chem., vol. 33, p. 55.

WILLIAMS, R. R., AND J. K. CLINE 1936 Synthesis of vitamin B_1. J. Am. Chem. Soc., vol. 58, p. 1504.

THE EFFECT OF YEAST AND OF THIAMIN ON THE PRODUCTION OF LOW IODINE GOITER[1]

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(Received for publication September 15, 1938)

Carpenter and Sharpless ('37) have recently reported rather startling findings concerning a heat labile factor, not vitamin B_1, in dried brewer's yeast capable of decreasing the size and increasing the iodine concentration of the thyroid glands of rats on a low iodine, goiter producing diet. In three groups of rats all receiving diets of equal iodine content (0.0038%) one group was fed 5% dried brewer's yeast, the second group 5% autoclaved yeast and the third 5% autoclaved yeast plus vitamin B_1 as a wheat germ concentrate. The results showed that the first group, which had received the untreated yeast, had smaller thyroids and considerably more thyroid iodine than did the animals in the other two groups.

These same workers also concluded that the goiter observed in animals on a low iodine, vitamin B_1-deficient diet can be prevented either by iodine or by vitamin B_1. The vitamin B_1-free diet containing 0.0038% iodine which they used, produced definite thyroid enlargement and a histological picture of goiter whereas if this diet were supplemented either with iodine or with a vitamin B_1 concentrate, the thyroids were smaller in size and histologically normal.

The experiment reported here was set up to test the conclusions of Carpenter and Sharpless by a somewhat different mode of attack. Young rats, averaging 75 gm. in weight, were

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[1] Aided by a grant from the Committee on Scientific Research of the American Medical Association.

placed on a low iodine, vitamin B_1-deficient diet for a preliminary period of 7 days. At this time all of the animals had ceased to grow and began to lose weight. They were divided into groups of ten, equally distributed as to sex, placed on the diets shown in table 1, and continued on experiment for 6 weeks, a period similar to that used by Carpenter and Sharpless. The technic devised by Remington ('37) was then used, wherein the status of the thyroid gland is quantitatively measured by the determination of three criteria: weight of gland in relation to body weight, dry matter and iodine content of the gland. The results are shown in table 1.

TABLE 1

	GROUP			
	I Low-iodine adequate B_1 (yeast)	II Low-iodine vitamin B_1-free	III Low-iodine adequate B_1 (thiamin)	IV Adequate iodine vitamin B_1-free
Diet				
Sucrose (%)	68	68	68	68
Wheat gluten (%)	18	18	18	18
Dried yeast (%)	5	..	..	..
Autoclaved yeast (%)	..	5	5	5
Pig liver (%)	2	2	2	2
Salt mixture no. 351 (%)	2	2	2	2
Cottonseed oil (%)	5	5	5	5
Iodine (γ %)	..	..	..	50
Vitamin supplements (daily)				
Carotene (IU)	4	4	4	4
Viosterol (IU)	4	4	4	4
Thiamin (γ)	..	..	15	..
Results				
Weight gain (gm.)	79.9	−18.1	72.3	−8.2
Thyroid weight (mg.)	20.5	8.2	17.2	7.8
Thyroid weight per 100 gm. body weight (mg.)	13.0 ± 0.52	12.8 ± 0.41	11.4 ± 0.23	11.0 ± 0.39
Dry matter (%)	23.6	23.3	22.2	35.2
Iodine, dry basis (%)	0.049	0.088	0.072	0.283

The animals of groups II and IV all showed polyneuritic symptoms typical of vitamin B_1 deficiency at least once during the experimental period. Each time these symptoms occurred the animal was given sufficient thiamin (6 γ) orally to relieve the paralysis and allow a slight gain in weight for approximately 5 days. In this manner the animals were all maintained on their respective diets the same length of time.

It might be well at this point to note that although the basal diet used by Carpenter and Sharpless reputedly contained less than 20 γ of iodine per kilogram yet their values for size, dry matter and iodine content of glands of rats maintained on this diet were within the normal range as determined by Remington, Remington and Welch ('37). The inference must be drawn that these animals had received prophylactic amounts of iodine from some source. Furthermore, the diets to which iodine was added furnished prodigious quantities of this element in relation to the minimal protective dose of 1 γ to 2γ per day as established by Levine, Remington and von Kolnitz ('33). Assuming an average daily food consumption of 10 gm. the diets containing 0.0038% and 0.019% iodine would supply 380 γ and 1900 γ per rat per day or from 200 to 1000 times the amount necessary for normal thyroid development. Apparently these investigators were in no case dealing with low iodine goiter. Their histological sections tend to confirm this opinion.

In the present experiment at autopsy the thyroid glands of all group IV animals (those receiving the adequate iodine, vitamin B_1-free diet) were obviously normal on gross inspection, viz., very pale in color and firm in texture. In contrast, the glands from all the animals in the other groups were definitely goitrous in that they were apparently enlarged and were dark red in color with considerable engorgement and distension of the blood vessels leading to the gland. The iodine deficiency in the diets employed, while producing definite changes in the dry matter and iodine content of the glands, was not sufficiently drastic to cause significant increases in thyroid weights (Remington and Levine, '36).

It is apparent, therefore, under the conditions of the present experiment, that dried brewer's yeast is ineffective in influencing the size or iodine content of thyroid glands of rats fed a low iodine, goiter producing diet. The yeast fed rats possessed thyroids equal in size, dry matter and iodine content to those of the animals receiving autoclaved yeast or autoclaved yeast plus thiamin.

Furthermore, the data show clearly that vitamin B_1 cannot substitute for iodine in preventing low iodine goiter. Thyroids of rats on a low iodine, vitamin B_1-free diet supplemented by minimal amounts of iodine (approximately 2 to 3 γ per day) proved to be normal in every respect while the glands of rats on the same diet supplemented by vitamin B_1 (15 γ thiamin daily) presented a picture of true goiter. The glands differed in no way from those of animals given the same basal diet with no vitamin B_1 supplement. The percentage of dry matter was low, the iodine content was low and the macroscopic appearance was typical of hyperplasia.

LITERATURE CITED

Carpenter, M. D., and G. R. Sharpless 1937 A study of the effect of vitamin B and iodine on the weight, iodine content and structure of the thyroid gland of the rat. J. Nutrition, vol. 13, p. 235.

Levine, H., R. E. Remington and H. von Kolnitz 1933 Studies on the relation of diet to goiter. II. The iodine requirement of the rat. J. Nutrition, vol. 6, p. 347.

Remington, R. E. 1937 Improved growth in rats on iodine deficient diets. J. Nutrition, vol. 13, p. 223.

Remington, R. E., and H. Levine 1936 Studies on the relation of diet to goiter. III. Further observations on a goitrogenic diet. J. Nutrition, vol. 11, p. 343.

Remington, R. E., J. W. Remington and S. S. Welch 1937 The thyroid gland of the normal rat: size, dry matter and iodine content. Anat. Rec., vol. 67, p. 367.

THE ACID SOLUBLE PHOSPHORUS CONTENT OF MUSCLE OF RATS UNDER VARIOUS DIET MODIFICATIONS[1]

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ONE FIGURE

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Previous reports from this laboratory have indicated that the content of acid soluble phosphorus in muscular tissue of the rat is not constant over the life of the animal (Struck and Visscher, '35; Bartoli, Cohen and Struck, '37). Numerous other chemical changes relating to growth and age have appeared in the literature reviewed by Pincussen, Reed and Visscher ('38) in a report on the content of acid soluble phosphorus in human tissues at various ages.

The report by Struck and Visscher of a decrease in the content of acid soluble phosphorus in old animals was partially confirmed in another colony that was maintained for 11 months and sampled at regular intervals. However, since certain confusing factors cast some doubt on the conclusions, a third large colony was maintained for 65 weeks.

Since there is no clear means of differentiating between the chemical changes incident to growth and those due to aging, an attempt was made to determine whether factors that might influence growth in either direction would have any influence on the concentration of acid soluble phosphorus. The animals were Wisconsin strain white rats, 3 weeks old when placed on the stock laboratory diet.

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[1] Part of the expenses of this investigation was borne by grants from the Wander Company, from the Nutrition Research Laboratories and from the Graduate School Research Fund of the University of Illinois.

At the age of 13 weeks the animals were placed on the various special diets noted below. A group of ten animals, selected at random from the colony, was killed at this time and analyses for total acid soluble phosphorus made on gastrocnemius muscle and liver. Seven groups were disposed as follows:

Group 1. Controls: stock diet only. Ca and P content 0.5 to 0.6% with Ca: P ratio approximately 1.

Group 2. High phosphorus diet. Sufficient disodium phosphate was added to the stock diet to increase the phosphorus content to 1.25 to 1.50%. The Ca: P ratio varied from 0.33 to 0.41.

Group 3. Stock diet fortified with vitamin B_1 [2] in the form of Tiki-tiki extract.

Group 4. Diet 2 with the same vitamin B_1 supplement as for group 3.

Group 5. Stock diet; growth hormone [2] administered intraperitoneally.

Group 6. Diet 2, with the same dosage of growth hormone as for group 5.

Group 7. Diet 4, with the addition of vitamin D of such concentration that the daily dose, given by stomach tube, was 2½ units per rat.

The rats were kept on the various diets from the age of 13 weeks to the age of 65 weeks, when the experiment was terminated. Analyses for total acid soluble phosphorus of muscle and liver were made on all animals. The results were shown in table 1 and figure 1. At 43 weeks of age ten rats from each group were killed and analyzed.

The technic for analyses was: The rats were anaesthetized with ether, the gastrocnemii and liver were quickly excised and immediately dropped into liquid air. Weighed samples of muscles and liver were ground to powder with mortar and pestle cooled with liquid air, and the powder then extracted with a measured quantity of 0.25 N trichloracetic acid.

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[2] Antuitrin Growth was generously supplied by Parke, Davis and Company; vitamin B_1 in the form of Tiki-tiki extract, by the Harris Laboratories.

Aliquot parts of the filtered extract were ashed in micro-Kjeldahl flasks with concentrated H_2SO_4 and 30% H_2O_2. After boiling with water to decompose excess H_2O_2, phosphorus was determined by a modified Bell-Doisy method.

The growth curves showing the influence of the dietary modifications were omitted in order to conserve space, but

TABLE 1

Total acid soluble phosphorus muscle (moist tissue), third colony

AGE	MEAN P CONTENT	STANDARD DEVIATION	STANDARD ERROR
	mg.%		
Group 1			
3 months	125	9.92	3.14
10 months	155	3.95	1.49
16 months	113	14.60	4.40
Group 2			
3 months	125	9.92	3.14
10 months	159	6.91	2.44
16 months	129	10.07	3.36
Group 3			
3 months	125	9.92	3.14
10 months	156	8.04	2.84
16 months	137	13.55	3.29
Group 4			
3 months	125	9.92	3.14
10 months	146	12.10	4.03
16 months	132	12.87	4.86
Group 5			
3 months	125	9.92	3.14
10 months	144	6.83	2.41
16 months	143	13.90	4.64
Group 6			
3 months	125	9.92	3.14
10 months	148	10.23	3.41
16 months	132	15.39	5.13
Group 7			
3 months	125	9.92	3.14
10 months	143	13.76	4.59
16 months	126	19.76	6.25

will be made available to anyone interested. In all groups the growth rate was inhibited compared with the controls, except in those receiving growth hormone.

The values obtained for the phosphorus content of the muscles of rats of the third colony are shown in table 1. Probability factors were calculated according to the method given by Fisher, but are not included for lack of space.

Data were also obtained for total acid soluble phosphorus content of liver at the same intervals, but are not included since they did not show consistent changes. In general, the results for the livers followed the same trend as the muscles, but gave changes which were significant with considerably less regularity than the muscles.

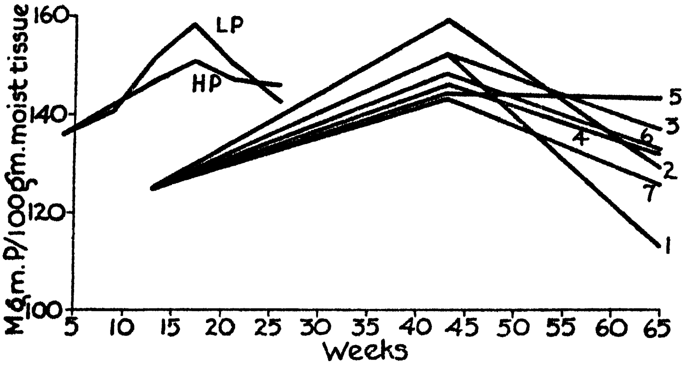

Fig. 1 L.P. (low P diet) and H.P. (high P diet) represent changes in acid-soluble P in muscle of rats in colony IV. Graphs 1 and 7 inclusive represent changes in acid soluble P in muscles of rats in colony III. 1, normal controls; low P diet. 2, high P diet. 3, no. 1 diet + vitamin B_1. 4, no. 2 diet + vitamin B_1. 5, no. 1 diet + growth hormone. 6, no. 2 diet + growth hormone. 7, no. 4 diet + vitamin D.

The analytical data were then studied in an effort to correlate the changes in phosphorus content with the various diets. The results of this study are not included here, but may be summarized briefly.

In all groups the acid soluble phosphorus content of muscle increased up to 42 weeks and decreased thereafter except in group 5 (low P diet + growth hormone). The differences at 43 weeks were not statistically significant. At 65 weeks, the differences were significant between groups 1 and 5. Apparently the growth hormone retards the decrease in acid soluble phosphorus on a low P diet.

Cole and Koch ('31) have studied the phosphorus fractions of striated muscles of rats and have found that there is a rapid increase in creatine phosphate content from birth to 2 or 3 weeks after weaning, a change which may be due to the relatively great increase in exercise during this period. They did not find any significant change from the maximum during a total period of observation up to the 168 days of age.

It seemed possible that this early increase had been missed in our series. In an effort to check this point another series of experiments was carried out on a fourth colony. The rats were obtained from the same source as those in the other experiments. At weaning they were placed on the stock diet as above for 1 week, after which ten of each sex were selected at random and basic control analyses were made. Half of those of each sex were then placed on a diet prepared by adding disodium phosphate to the stock diet in quantity sufficient to bring the phosphorus content to 1.25 to 1.5%.

As already described, analyses of the muscles of the rats were made on ten males and ten females of each group at the ages of 28, 60, 90, 120, 150 and 180 days.

The growth curves of the animals of the fourth colony showed that the high phosphorus diet caused a definite retardation of growth in males, although not in the females. The results of the analyses are summarized in table 1 (fourth colony) and figure 1. The acid soluble phosphorus contents of the muscles of rats on both diets, when calculated as per cent of moist tissue in both groups increased significanty to a maximum at 120 days of age then slowly decreased, the final value at 180 days being higher than the control at 28 days.

Tables showing comparison of water, acid-soluble phosphorus and nitrogen content of the muscles of the two groups at monthly intervals were prepared, but are omitted for lack of space.[3] It was observed that there are significant differences between the two groups only in random instances, leading to the provisional hypothesis that during the first 6 months of life the addition of sodium phosphate to the diet in the amounts used here has no effect on these constituents.

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[3] Complete data are available for those interested in details.

In figure 1 the two small curves labeled L.P. and H.P. confirm Cole and Koch in their report of an early increase in the muscle content of acid soluble phosphorus. However, there then occurred a decrease which placed the values at the end of 25 weeks somewhere near those for groups 1 and 2 and in the third colony, as shown in the same figure. However, the values in these earlier observations were unquestionably increasing. Furthermore, the initial determinations made at 13 weeks of age were very much lower than occurred at any time in the fourth colony. Both lots were treated similarly and were of the same strain. The only explanation

TABLE 2

Total acid-soluble phosphorus content of muscles of rats (moist tissue), fourth colony

AGE	DIET	MEAN PHOS.	NUMBER OF ANALYSES	STANDARD DEVIATION	DIFFERENCE [2]
days		*mg.%* [1]			
28	SD	136	17	9.6	..
60	SD	141	15	9.9	5
	HP	142	16	11.5	6
90	SD	151	14	9.4	15
	HP	147	13	9.2	11
120	SD	158	15	6.6	22
	HP	151	14	7.7	15
150	SD	150	15	9.1	14
	HP	147	17	5.7	11
180	SD	143	17	5.8	7
	HP	146	18	7.6	10

SD = stock diet, Ca: P ratio 1.
HP = high phosphorus diet.

[1] Includes values for right and left muscles and for males and females.

[2] Differences from value at 28 days of age.

that can be offered at present is that there is a seasonal influence. All rats in the third colony were born August 18, 1935. Those in the fourth colony were born March 18, 1937. It will be necessary to investigate further the possible seasonal influence.

SUMMARY

1. The acid soluble phosphorus content of skeletal muscle of white rats in the third colony on all dietary modifications used showed a marked increase from 13 weeks to 43 weeks.
2. The high phosphorus diet produced the greatest increase. The other diets retarded the increase when compared to the normal diet, but the differences were not statistically significant.
3. From 43 to 65 weeks there was a definite decrease in acid-soluble phosphorus content which was most pronounced on the normal diet and definitely retarded in all other groups. Normal diet plus growth hormone prevented the decline practically completely. Again differences were not statistically significant except in this one instance.
4. In the fourth colony there was found an increase in the acid soluble phosphorus content of muscle up to 17 weeks, which was retarded in this case by the high phosphorus diet. From 17 to 25 weeks the decrease found on the normal diet was retarded by high phosphorus diet.

LITERATURE CITED

BARTOLI, A. J., J. L. COHEN AND H. C. STRUCK 1937 A study of the total acid-soluble phosphorus in skeletal muscle of rats. Proc. Am. J. Physiol., p. 11.

COLE, V. V., AND F. C. KOCH 1931 A study of phosphorus distribution in rat striated muscle as influenced by age. J. Biol. Chem., vol. 94, p. 263.

FISCHER, R. A. 1928 Statistical methods for research workers. 2nd. ed., Oliver and Boyd, Edinburgh.

PINCUSSEN, L., C. I. REED AND M. B. VISSCHER 1938 The relationship of age to concentration of acid soluble phosphorus in human tissues. Ann. Int. Med., vol. 12, p. 59.

STRUCK, H. C., AND M. B. VISSCHER 1935 Studies on changes with increasing age in the phosphorus fractions of various tissues of the rat. Proc. Am. J. Physiol., vol. 1, p. 128.

THE EFFECTS OF HIGH HUMIDITY ON SKIN TEMPERATURE AT COOL AND WARM CONDITIONS [1]

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The importance of humidity in thermal reactions of the body has never been satisfactorily evaluated. Yaglou ('37) states that "under ordinary indoor conditions during the heating season, variations in humidity are relatively unimportant" and that "practically all physical, physiologic and psychologic tests have proved to be negative or inconclusive." Wiley and Newburgh ('31) found no change in skin temperature when subjects were exposed to a relative humidity varying from 20 to 80% at a room temperature of 28°C. In ten subjects exposed to ordinary room temperatures (20 to 22.5°C.) Talbot ('31) observed no consistent changes in skin temperature when the relative humidity varied from 31 to 90%. Winslow, Herrington and Gagge ('37) noted that skin temperatures were in general the same for both low and high humidities. On the other hand, Muira ('31) observed that the skin temperature of the forehead and over the carotid artery tended to vary directly with the relative humidity of the air at temperatures between 60° and 80°F. although the variation was small. In addition, Phelps and Vold ('34) noted a slight positive correlation between the temperature of the forehead and cheeks and the relative humidity during the closed window season.

In view of the inconsistency in results of previous researches the subject was investigated in normal individuals as a part of a physiologic study of thermal reactions from the standpoint of their autonomic reactivity.

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[1] This investigation was aided by a grant from the Rockefeller Foundation.

The study was carried out on ten normal male subjects who lived at the institution throughout the duration of the tests. On each experimental day each participant came from his bed under fasting conditions to the temperature laboratory where he disrobed and lay down upon a cane-woven bed without any coverings. Immediately after reclining and every 30 minutes thereafter over a period of 3 hours, measurements were taken of the skin temperatures thus giving a total of seven series of readings. Skin temperatures were determined at ten representative areas on the right anterior surface of the body, one side being considered sufficient because of the high degree of bilateral symmetry of skin temperatures (Freeman, Linder and Nickerson, '37). All readings were taken by means of copper-constantin thermocouples.

The effects of humidity were studied by a comparison of the levels of skin temperatures observed on 2 days at the same environmental temperature, the first day having a constant low humidity of 20% and the second day a relative humidity which increased from 20 to 90%. The procedure was carried out at two environmental temperatures, 24°C. and 32°C. conditions which are ordinarily characterized by a cooling of the skin and a warming, respectively. For the purposes of this experiment, therefore, 4 days were utilized. The actual environmental conditions for the days with changing humidity are shown in table 1. On each humid day the first two series of measurements were made under the same conditions as on the previous dry day in order to be sure that the subjects started at approximately the same level. The rate of increase in relative humidity on the 2 humid days is quite similar so that a comparison of the results obtained at the different temperatures is possible. The air velocity is essentially the same for the 4 days, the average level being 41 feet per minute.

The behavior of the skin temperatures at 24°C. and 20% R.H. are shown in table 2 in the columns headed 24°, 'Dry.' Here are tabulated the means of the ten subjects for the seven series of measurements at the various skin points. Since they serve as a basis for a comparison of the data obtained on the humid days, they are presented in full though in themselves they show little that is new. In summary, it may be said that there is a downward trend in all the skin temperatures, the fall being more marked and prolonged on the extremities. A stable level is reached on the trunk after 1 hour of exposure and on the extremities 1½ hours later.

TABLE 1

Means of levels of relative humidity attained at 30-minute intervals at environmental temperatures of 24°C. and 32°C.

Time of exposure Minutes	*Relative humidity (D.B. 24°C.)* %	*Relative humidity (D.B. 32°C.)* %
0	22.8	19.1
30	22.4	21.3
60	49.6	47.9
90	77.6	71.8
120	87.7	86.9
150	90.0	90.4
180	90.5	90.2

As the relative humidity is increased from 20%, after the first 30 minutes of exposure there is observed a divergence of trends from those seen under conditions of constant low humidity so that the fall in skin temperature is markedly lessened. This results in the values for skin temperature being higher than those obtained at the corresponding time at 20% R.H. The difference in effects is shown in table 2 in the columns marked 24° 'Humid.' The first two readings on the 'dry' and 'humid' days are obtained under the same environmental conditions. The slight deviations present are probably due almost entirely to the innate variability of the organism. The third reading on the 'humid' day at which the relative humidity has been increased to 50% begins to show a difference in effect from that of the corresponding value at

TABLE 2

Mean skin temperatures (0°C.) of ten normal subjects at environmental temperature of 24°C. with high and low humidity and 32°C. with high and low humidity

			TIME OF EXPOSURE IN MINUTES							AVERAGE DIFFERENCE BETWEEN TERMINAL READINGS
			0	30	60	90	120	150	180	
Forehead	24°	Dry	31.9	32.4	32.4	32.2	31.9	31.8	31.9	0.5
		Humid	31.9	32.7	32.7	32.8	32.6	32.6	32.4	
	32°	Dry	33.5	34.6	34.6	34.8	34.9	35.0	34.9	0.2[1]
		Humid	33.7	34.8	35.2	35.4	35.3	35.3	35.1	
Chest	24°	Dry	31.9	31.5	31.3	31.1	31.2	31.2	31.3	0.4
		Humid	32.0	31.7	31.7	31.9	31.7	31.7	31.7	
	32°	Dry	33.7	34.2	34.3	34.6	34.6	34.8	34.8	0.1[1]
		Humid	33.7	34.5	34.9	35.1	35.1	35.0	34.9	
Abdomen	24°	Dry	32.6	32.0	31.8	31.5	31.5	31.5	31.4	0.3
		Humid	32.8	32.2	32.0	32.0	32.0	31.9	31.7	
	32°	Dry	34.1	34.3	34.3	34.5	34.7	34.7	34.7	0.2[1]
		Humid	34.2	34.5	34.8	35.1	35.0	34.9	34.9	
Upper arm	24°	Dry	32.4	32.0	31.6	31.4	31.2	31.1	31.0	0.5
		Humid	32.5	32.1	31.9	31.9	31.8	31.7	31.5	
	32°	Dry	34.0	34.3	34.3	34.6	34.7	34.8	34.7	0.2[1]
		Humid	34.0	34.5	35.0	35.2	35.1	35.1	34.9	
Lower arm	24°	Dry	32.2	31.6	31.2	30.5	30.2	29.9	29.8	0.9
		Humid	32.3	31.9	31.5	31.2	31.0	30.9	30.7	
	32°	Dry	33.9	34.1	34.3	34.5	34.6	34.6	34.7	0.4
		Humid	33.7	34.3	34.9	35.2	35.3	35.2	35.1	
Thumb	24°	Dry	28.5	27.7	27.3	26.5	25.9	25.6	25.5	2.2
		Humid	29.4	28.4	28.0	27.9	28.0	27.8	27.7	
	32°	Dry	31.7	33.3	33.7	33.4	33.6	33.6	33.5	1.8
		Humid	31.4	33.9	34.8	35.3	35.4	35.3	35.3	
Finger	24°	Dry	23.9	23.9	24.4	23.4	23.1	23.0	22.9	3.3
		Humid	25.6	25.2	24.9	25.8	26.4	26.4	26.2	
	32°	Dry	29.0	32.9	33.4	32.9	33.0	32.8	32.9	2.6
		Humid	29.6	33.5	34.9	35.4	35.5	35.6	35.5	
Thigh	24°	Dry	31.3	30.8	30.5	30.3	30.1	29.9	29.9	0.6
		Humid	31.6	31.0	30.9	30.9	30.8	30.7	30.5	
	32°	Dry	33.0	33.7	33.9	34.0	34.1	34.2	34.2	0.5
		Humid	33.3	34.0	34.5	34.8	34.8	34.8	34.7	
Lower leg	24°	Dry	31.3	30.5	30.0	29.6	29.4	29.2	29.2	0.9
		Humid	31.5	30.9	30.6	30.6	30.4	30.2	30.1	
	32°	Dry	33.1	33.6	33.6	33.8	34.0	34.0	34.0	0.5
		Humid	33.4	33.9	34.2	34.6	34.6	34.5	34.5	
Foot	24°	Dry	28.7	27.4	26.8	26.2	25.7	25.5	25.2	2.0
		Humid	29.5	28.3	27.9	27.8	27.7	27.5	27.2	
	32°	Dry	31.1	31.1	31.3	31.3	31.4	31.2	31.2	3.4
		Humid	30.7	31.2	32.3	33.6	34.3	34.6	34.6	

[1] Denotes non-significance.

20%, a variation which becomes more marked as the humidity increases. The maximum deviation from the corresponding value at 20% is obtained on the trunk when the relative humidity has reached 78% (fourth reading) after which there is a slight decline in the difference. On the hand, the effect of the increased humidity is much more marked than on the trunk and reaches a maximum difference at the sixth reading where the relative humidity is 90% after which the deviation from the corresponding value at 20% relative humidity is maintained.

It is evident, then, that the effect of increasing the humidity is to offset to a varying degree the cooling ordinarily observed at the same environmental temperature with a low humidity. This diminution of heat loss is noticeable when the realtive humidity has been increased to only 50% and reaches a maximum at higher levels of humidity, 78% on the trunk and 90% over the rest of the body. Following this increase in skin temperature, the thermodynamic situation is changed, and as a result of further loss through the channels of radiation and convection, the surface of the trunk and upper extremities tends to cool despite the maintenance or even increase of the high level of humidity. The difference in trends between the dry and humid days at 24° have been analyzed by the method of the analysis of variance and have been found to be statistically significant at all points but the abdomen.

A quantitative estimation of the effects of humidity on skin temperature is shown in table 2 in the column containing the differences between the terminal readings. For this analysis the averages of the last series of measurements on the dry and humid days have been taken in order to afford a comparison of the skin and body temperatures at 20% and 90% relative humidity after a sufficient period of exposure to allow thermal equilibrium to take place. It is evident in all instances that the skin temperatures are higher at 90% relative humidity than at 20%. These differences are all statistically significant. The average elevation of skin temperature varies topographically on the body surface, the extremities showing a much greater effect than the trunk. It should be noted that these differences should not be taken at their exact numerical value in view of the slight variation in the initial readings between the two experimental days and the tendency for the final skin temperatures to decrease slightly on the humid day. Nevertheless the effect of high humidity on skin temperatures is sufficiently marked, particularly on the hands and feet, so that there can be little question as to its physiologic significance.

Turning now to the effect of humidity at the higher temperature, it may be seen that under constant environmental conditions of 32°C. and 20% relative humidity (table 2, 32° 'Dry') the skin temperature rises, slightly on the trunk and markedly on the extremities. The elevation of temperature on the trunk reaches its maximum level in 2 hours while on the extremities, the highest point is attained in 30 minutes. All of the values are, of course, at a much higher level than the corresponding ones at 24°C. and 20% relative humidity.

When the relative humidity is raised, the same phenomenon is observed as at 24°C. The skin temperature again increases all over the body, slightly on the trunk and to a greater extent on the extremities (table 2). This increase is an augmentation of an already existing heating trend rather than lessening of a cooling trend as at 24°C. On the trunk and upper extremities, the effect of humidity reaches its maximum after 90 minutes of exposure at 72% R.H. when as seen at the lower temperature a new equilibrium is established and the magnitude of the increase in skin temperature diminishes. On the hands and feet, however, the effect of humidity reaches its maximum usually when 90% R.H. has been first attained, 2.5 hours after first exposure. The differences in the heating trends between the dry and humid days have been found to be statistically significant by the method of the analysis of variance and in all instances except over the forehead and chest.

The changes in skin temperature at 32°C. as a result of the introduction of high humidity have been computed in the same way as at 24°C. In table 2, it is seen that under the humid conditions the values are again increased with a similar degree of variation over the different parts of the body surface, the extremities again showing the greatest effect. The differences are not significant over the forehead, trunk and upper arms. A comparison of the increases in temperature at 32°C. with those at 24°C. brings forth the fact that the values at the warmer temperature are not higher as Miura ('31) has stated but are even slightly lower. In short, the effects of high humidity on skin temperature are quite similar irrespective of the fact whether the environmental condition is one where there is cooling or warming of the surface of the body. This is true at least within the limits of this study.

A consideration of these data reveals several interesting facts. First, increasing humidity at these air temperatures causes significant elevations in the temperature of the skin. The effect is noted when the humidity is increased from 20 to 50% and becomes more marked as the humidity is increased to 90%. In general, this result may be ascribed to the inhibition of evaporation of perspiration by the increased atmospheric moisture (Wiley and Newburgh, '31) and the negation of the heat loss which ordinarily follows upon this function.

Second, the increase in skin temperature thus observed is much more marked on the extremities than on the trunk. This may be due to two factors. The evaporation per unit surface is ordinarily much greater on the extremities than on the trunk (Kuno, '30). The neutralization of this cooling effect may be expected therefore to produce a greater rise in temperature at the distal portions of the body. A second factor is found in the relationship between the saturation vapor pressures at the temperatures of the skin and of the air.

Third, high humidity at the two environmental temperatures has resulted in increases in temperature which have been quite similar (or even less at 32°C.) for corresponding points on the skin.

Thermodynamic analysis

Following Winslow, Herrington and Gagge ('37) we analyze the heat exchange of the body into four components. Denoting metabolism by M, the rate of heat loss suffered through radiation and convection by C, the rate of heat loss suffered by the cooling of the tissues by S and the rate of evaporation by E the following equation holds:

$$M + S = E + C. \qquad (1)$$

According to the cited authors, metabolism is practically constant. It is reasonable to assume that the data of table 2 were obtained after a state of thermal equilibrium has been reached, so that the term S can be neglected.

While investigating the rate of heat loss through evaporation, we shall consider the skin of the human body as a water surface. However, we have to keep in mind that this is only a rough approximation, which applies only when the humidity is high. The ability of the human body to regulate its heat exchange depends primarily on the fact that it is able to act not as a water surface. When the humidity is low, the evaporation is less than that of a water surface, because only as much sweat is produced as is necessary for the maintenance of a heat equilibrium. This condition is described in the language of Winslow, Herrington and Gagge ('37) by saying that the wettedness of the body is less than 100%. However, if the humidity of the surrounding air is high, the excreted sweat cannot evaporate at once, the body becomes wet and acts like a water surface. Hence the following equation holds:

$$E = k_1 (p_T - p_{T_w}) T, \qquad (2)$$

where k_1 is an appropriate constant (depending on the air velocity), T is the absolute temperature of the skin, T_w is the wet bulb temperature and p_T the vapor pressure at absolute temperature T. It is easy to see without any calculations that if T_w is changed then the percental decrease in E will be much larger when T is near to T_w than otherwise. For small intervals and under 40°C., $p_T - p_{T_w}$ can be assumed to be proportional to $T - T_w$; hence $\frac{\partial E}{\partial T_w}$, the change of evaporation rate for the change of T_w by 1°C. is constant.

If, for example, $T_1 = 33°C.$, $T_2 = 25°C.$ and $T_w = 21°C.$ then the rates of evaporation at the points 1 and 2 compare like 3.9 to 1. The increase of T_w from 21°C. to 22°C. causes a decrease by the same amount in both cases, hence is much more significant for point 2 than for point 1.

As soon as the humidity reaches a high degree, as in the experiment, the evaporation slows down markedly at all points where the temperature is near air temperature. Since heat loss through convection and radiation does not increase unless the temperature of the skin increases, it is to be expected that the points of originally low temperature will warm up most. It happens that these points are on the extremities. The increases in temperature at various points of the body look quite proportional, but this is so because the orginal temperatures on the 2 dry days are similarly distributed. That is, if one point is warmer than the other on the cold dry day, then it is still warmer on the hot dry day. There are, however, exceptions; e.g., the foot point, and in these exceptional cases the proportionality breaks down. However, if we arrange the dry day mean temperatures in the order of magnitude, the correlation between dry day temperature and rise due to humidity is conspicuous.

SUMMARY

The effects of high humidity upon skin temperature were studied in ten normal subjects at environmental temperatures of 24°C. and 32°C. The variation due to humidity was observed by a comparison of the levels of surface temperatures when the subjects were exposed for 3 hours in a nude fasting state to low relative humidities (20%) and to increasing humidity (up to 90%) on 2 days at each environmental temperature. At 24°C., it was found that the cooling of the skin ordinarily observed at 20% relative humidity was partially inhibited by high humidity so that its temperature rose. At 32°C. the heating of the skin found at 20% relative humidity was increased by high humidity. The rise in temperature over the trunk was slight but on the extremities there was an increase of 2 to 3°C. as the result of increasing the relative humidity to 90%. The increase in skin temperature as the result of high humidity at 32°C. was no greater, in fact slightly less, than at 24°C.

LITERATURE CITED

FREEMAN, H., F. E. LINDER AND R. F. NICKERSON 1937 The bilateral symmetry of skin temperature. J. Nutrition, vol. 13, p. 39.

KUNO, Y. 1930 The significance of sweating in man. Lancet, vol. 218, p. 912.

MIURA, U. 1931 Humidity, skin temperature and sense of comfort. Am. J. Hyg., vol. 13, p. 457.

PHELPS, E. B., AND A. VOLD 1934 Studies in ventilation: Skin temperature as related to atmospheric temperature and humidity. Am. J. Pub. Health., vol. 24, p. 959.

TALBOT, F. B. 1931 Skin temperatures of children. Am. J. Dis. Child., vol. 42, part II, p. 965.

WILEY, F. H., AND L. H. NEWBURGH 1931 The relationship between the environment and the basal insensible loss of weight. J. Clin. Inves., vol. 10, p. 689.

WINSLOW, C.-E. A., L. P. HERRINGTON AND A. P. GAGGE 1937 Physiological reactions of the human body to various atmospheric humidities. Am. J. Physiol., vol. 120, p. 288.

YAGLOU, C. P. 1937 Physical and physiologic principles of air conditioning. J. Am. Med. Assoc., vol. 108, p. 1708.

THE EFFECT OF CHONDROITIN SULFURIC ACID ON GIZZARD EROSION AND GROWTH IN CHICKS

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These investigations were stimulated by the possibility that chondroitin sulfuric acid (hereafter abbreviated as CSA) might prevent gizzard erosions. This theory was based on our observation that CSA exhibited very similar properties to those described for the active portions of lung tissue by Bird, Kline, Elvehjem, Hart and Halpin ('36). Bird and Oleson ('38) later presented a preliminary report on CSA as the anti-gizzard erosion factor. Meanwhile our investigations had indicated that CSA was not effective. The diet used by Bird and Oleson was a modification of that which they had employed in previous studies. Since our results with CSA added to the older gizzard erosion producing diet (454) of the Wisconsin investigators were at variance with their findings when the modified diet was employed, we extended our experiments to include further studies on both the new and old gizzard erosion producing diets of the Wisconsin group, on ordinary commercial chick mash and on the gizzard erosion producing diet of Almquist and Stokstad ('37). In all, about 400 chicks were used in these investigations and the results have been subjected to statistical analysis.

METHOD AND DIETS

Barred Plymouth Rock chicks were used throughout these studies and were placed on the diets when 1 day old. The chicks were wing-banded, kept in temperature controlled brooders in an air-conditioned room, and allowed free access to feed and water. The chicks were individually weighed at 2 and 4 weeks, at which time they were sacrificed for inspection of the gizzards. In some of the first experiments, a group from each lot was sacrificed at earlier intervals. The gizzard erosions were graded by number as follows: 1, none; 2, slight; 3, marked and 4, severe. Statistical analyses were made in each group to determine the significance of weight differences and incidence of erosion. The mean erosion score was determined by adding the gizzard erosion grades for all chicks and dividing by the number; the probable error of the difference between the mean erosion score of the control and supplemented animals was then determined in the usual manner.

The basal diets employed are referred to in this report as Wisconsin diet 454 as given by Bird, Kline, Elvehjem, Hart and Halpin ('36), the new Wisconsin diet used by Bird[1] and Oleson ('38), and that of Almquist and Stokstad ('37) (abbreviated as A & S). When a vitamin K supplement was added to the latter diet, it consisted of a hexane extract of alfalfa meal dried on the basal ration in an amount equivalent to 2% of the original material. The new Wisconsin diet consisted of heated dextrin 64, heated purified casein 18, salts 5, dried brewer's yeast 1, liver extract 2, cod liver oil 1, and alfalfa leaf meal 10.

Both commercial[2] and purified CSA were used. Purification was accomplished by digestion with 2% sodium hydroxide and precipitation with 1 to 1½ volumes of alcohol. The precipitate was then dissolved in water and the CSA reprecipitated with 6 volumes of glacial acetic acid. The commercial product was found to contain about 28% glucuronic acid, the purified material about 33% glucuronic acid. Some protein and salts were present in the commercial product; the purified CSA was a biuret-free powder.

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[1] We wish to thank Dr. H. R. Bird for giving us the details of this diet in a personal communication.

[2] Obtained from the Wilson Laboratories, Chicago, Illinois.

RESULTS

The results of the first experiment in which Wisconsin diet 454 was used and 5% CSA was the supplement, are given in tables 1 and 2.

TABLE 1

Tests 28-W3 and 28-W8. Growth on Wisconsin diet 454 compared with supplement of 5% commercial chondroitin sulfuric acid

AGE OF CHICKS	BASAL DIET		BASAL DIET PLUS 5% CSA		DIFFERENCE OF MEANS	P.E. OF DIFFERENCE
	Number of chicks	Mean weight	Number of chicks	Mean weight		
weeks		*gm.*		*gm.*		
2	38	62.5	35	72.8	10.3	±1.56
3	10	89.9	10	116.3	26.4	±7.4
4	15	125.7	15	138.9	13.2	±6.5
5	14	154.3	10	192.9	38.6	±11.2

TABLE 2

Tests 28-W3 and 28-W8. Incidence and severity of gizzard erosions on Wisconsin diet 454 compared with supplement of 5% commercial chondroitin sulfuric acid

AGE OF CHICKS	BASAL DIET					BASAL DIET PLUS 5% CSA					DIFFERENCE OF MEANS	P.E. OF DIFFERENCE
	Gizzard erosion score[1]				Mean erosion score	Gizzard erosion score				Mean erosion score		
	1	2	3	4		1	2	3	4			
weeks												
2	1	3	5	1	2.6	5	2	1	2	2.0	0.60	±0.31
3	1	4	3	2	2.6	3	5	1	1	2.0	0.60	±0.27
5	3	4	4	3	2.43	2	3	4	1	2.40	0.03	±0.25

[1] Figures show number of chicks receiving each score.

The growth of these chicks (table 1) was significantly improved by the addition of CSA. In each weighing except that carried out on the fourth week, the difference between the mean weights of the chicks on the basal ration and those receiving CSA was statistically significant. It may be noted that growth on the Wisconsin diet 454 was rather poor when compared to that attained on good commercial chick mash.

A deficiency of other factors than that which prevents gizzard erosion was indicated inasmuch as the A & S diet (table 3) gave good growth while producing gizzard erosions of high severity.

Table 2 presents the individual erosion score of each chick as well as the mean erosion scores, their differences and their probable errors. This table illustrates clearly the fallacy of judging by inspection the effectiveness of a particular factor. It will be noted that in the chicks killed at 2 weeks, on the basal diet 40% show slight or no erosion and 60% show marked or severe erosion, while when CSA is added 70% show slight or no erosion and 30% marked or severe erosion. This might seem to indicate that CSA reduced the incidence of erosion, although since two chicks on CSA showed severe erosion as compared with but one on the basal diet it would seem improbable even here that CSA could be a specific factor. However, the difference of the mean scores of the two groups is 0.60 with a probable error of 0.31 which indicates clearly that the scattering within the groups is so great that one cannot draw any conclusion relative to the effectiveness of CSA. This is confirmed again in the chicks killed at 3 weeks, while in those killed at 5 weeks the difference between the means practically disappears and the probable error remains large.

The majority of our observations are condensed in table 3. In the first group (32A), CSA was used to supplement an ordinary commercial chick mash which was adequate for growth and on which it was known that the incidence of gizzard erosion is very small. This experiment was carried out to determine whether CSA would show a growth promoting effect when added to an adequate diet such as it exhibited when added to the Wisconsin diet 454. No effect was observed.

In group 41H the experiment on the Wisconsin diet 454 was repeated, using both commercial and purified CSA. In this group of animals neither commercial nor purified CSA improved growth. The chicks receiving the supplement, in fact, gained less weight than those on the basal ration although the difference was not significant. When commercial CSA

TABLE 3

Tests upon growth promoting and erosion preventing activity of commercial and purified chondroitin sulfuric acid with commercial chick mash, two Wisconsin, and A & S gizzard erosion producing diets

TEST AND DIET	AGE OF CHICKS	BASAL DIET				BASAL DIET PLUS 3% CSA				STATISTICAL ANALYSIS			
		Number of chicks		Mean weight	Mean erosion score	Number of chicks		Mean weight	Mean erosion score	Difference of mean weights	P.E. of difference	Difference of erosion score	P.E. of difference
		Start	End			Start	End						
	weeks												
32A	2	40	35	107		40	40	105		2	± 2.2		
Chick mash	4	40	35	274		40	39	268		6	± 6.5		
	6	40	34	417	1	40	37	413	1	4	±12.4		
41H	2	16	16	93		17	17	89		4	± 2.8		
Wisconsin 454	4	10	10	209	1.2	17	16	188	1.6	21	± 7.8	0.4	±0.12
	2					16[1]	16	95		2	± 3.1		
	4					11[1]	10	192	1.3	17	± 7.1	0.1	±0.10
41K	2	19	19	79		18[1]	18	83		4	± 2.7		
New Wisconsin	4	19	19	146	1.2	18[1]	18	152	1.2	6	± 7.0	0.0	±0.08
41M													
A & S no	2	9	9	119		10	10	132		3	± 7.8		
vitamin K	4	9	2	218		10	7	290					
41N	2	10	10	123		10	10	120		3	± 8.8		
A & S plus	4	10	10	217	1.5	10	10	216	1.2	1	±13.9	0.3	±0.13
vitamin K	2					10[1]	10	119		4	± 7.5		
	4					10[1]	10	216	1.1	1	±13.5	0.4	±0.12
421													
A & S no	2	10	10	132		10	10	132		0	± 6.1		
vitamin K	4	10	5	249	3.8	10	6	274	3.7	25	±14.0	0.1	±0.71
42K													
A & S plus	2	10	10	125		10	10	127		2	± 4.9		
vitamin K	4	10	9	266	2.3	10	10	253	1.7	13	± 7.3	0.6	±0.18
42M	2	10	10	79		10	10	88		9	± 2.7		
New Wisconsin	4	10	10	116	1.9	10	10	126	1.5	10	± 5.2	0.4	±0.19
42-O	2	10	10	105		10	10	101		4	± 4.5		
Wisconsin 454	4	10	10	162	1.6	10	10	163	1.7	1	±10.0	0.1	±0.21

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[1] Purified chondroitin sulfuric acid used.

was used with the basal diet, the erosion score was higher and the difference is on the borderline of significance, but with purified CSA the difference disappears. In group 41K, chicks of the same age, weight, and source placed on the new Wisconsin diet and given a supplement of purified CSA failed to show any effect of CSA on growth or erosion.

In group 41M, the A & S diet was used without being supplemented with vitamin K. At 2 weeks the weight differences were not significant; at 4 weeks seven of the nine receiving the basal ration and three of ten receiving the basal ration plus 3% commercial CSA had died of the hemorrhagic diathesis induced, and the numbers are obviously too few for analysis. It may be said, however, that all the chicks remaining as well as those that died showed marked or severe erosion.

The A & S diet was supplemented with a vitamin K extract for group 41N and the mortality reduced to zero. No significant weight differences appear, and although the erosion score of the animals given CSA is lower than that of the chicks on the basal ration, the significance of the difference is questionable.

In groups 42I, 42K, 42M and 42-O, the experiments on the A & S diet with and without vitamin K and on the new and old Wisconsin diets were then repeated with a different lot of chicks. These experiments fully confirm those of the preceding groups. There are no significant weight differences, but CSA has a possibly significant effect upon gizzard erosion in group 42K (A & S diet plus vitamin K). It may be noted that enough chicks survived on the A & S diet in the absence of vitamin K to permit analysis in this case, and that their gizzard erosion score is much higher than that produced by any other ration, although it is not influenced by CSA.

DISCUSSION

Our results may be summarized by saying that in experiments upon 397 chicks divided into eleven groups in which the effect of CSA as a supplement was statistically analyzed, CSA had a growth-promoting effect in one instance in chicks given the Wisconsin diet 454 and failed to promote growth in four other instances on either the old or new Wisconsin diets. The growth-promoting effect occurred in a group that showed a greater incidence of gizzard erosion than we were later able to obtain upon these diets but there was no significant effect upon gizzard erosion. It seems likely that some constituent of Wisconsin diet 454 varies in its nutritional value. CSA had a possibly significant growth-promoting effect in one instance (group 41M) when the A & S diet was given without additional vitamin K, but failed to promote growth significantly when this diet was given to another group and also failed to promote growth in two instances when the A & S diet was given with vitamin K. It may be noted that all chicks on the A & S diet without vitamin K showed the expected hemorrhagic lesions.

The effect of CSA on gizzard erosions was not indubitably significant in any group. In group 41H (Wisconsin diet 454), it appeared to increase the incidence of erosions, the difference in score being 0.4± 0.12; in group 41N (A & S diet plus vitamin K) the difference in erosion score favored CSA being again 0.4 ± 0.12 and in a second group on the A & S diet plus vitamin K CSA may have decreased erosion, the score difference being 0.6 ± 0.18. No differences that suggested a CSA effect appeared in any other group.

Our observations force the conclusion that CSA is not the gizzard erosion preventing factor. The fact that in some instances CSA seems to have a growth-promoting effect and also seems to decrease the incidence of gizzard erosion slightly in some groups may permit us to suspect that this substance has a favorable effect upon the nutrition of the chick in the presence of certain dietary deficiencies. Further studies upon the addition of CSA to deficient diets are now in progress which support this suggestion. If a substance is to be regarded as a specific factor for the prevention of a given deficiency syndrome, it must give unequivocal results. Any possible effect of CSA upon gizzard erosion can only be regarded, in the light of our analysis, as secondary to a general improvement in nutrition. That CSA has a systemic effect has been shown by previous experiments of Crandall, Roberts and Gibbs ('32) and Crandall, Roberts and Snorf ('36) upon Eck fistula dogs and animals receiving cinchophen. That it is a dietary factor of importance for the chick and the rat will be reported in a separate communication.

An observation incidental to the main objective of this study concerns the differences between the unsupplemented A & S diet and the Wisconsin group diets in the production of gizzard erosion. In our studies it was found that the former diet permits substantially normal growth with a high incidence of gizzard erosions as compared with those of the Wisconsin investigators in which growth is greatly depressed and the incidence of gizzard erosion often relatively low. It should also be pointed out that the addition of vitamin K to the A & S diet produced a marked decrease in the incidence of gizzard erosions, and since the vitamin K was added as a hexane extract supports the contention of these investigators that the erosion preventing factor is ether soluble. We do not, however, offer this as a final conclusion since it seems to us entirely possible that the presence of a hemorrhagic diathesis as in vitamin K deficiency may increase the extent of the gizzard lesions even though there is no change in the erosion preventing factor in the diet. In other words, it is conceivable that vitamin K may decrease the extent of gizzard erosion merely by preventing hemorrhage.

An interesting condition which occurred almost universally in chicks on the Wisconsin diets was a distension and darkening of the cecum. In general this seemed to be somewhat less in the birds receiving CSA, although the difference did not appear in every group. Again one is inclined to consider a non-specific improvement in nutrition rather than a specific effect of CSA upon this condition of megacecum.

SUMMARY

Studies upon nearly 400 chicks given various gizzard erosion producing diets fail to reveal any unequivocal protective action of commercial or purified chondroitin sulfuric acid. This substance does appear, under certain conditions, to have a favorable effect upon growth. It is suggested that any slight influence upon gizzard erosion may be the result of a general nutritional improvement rather than the action of a specifically protective substance. Incidental observations upon chicks receiving gizzard erosion producing diets are discussed.

LITERATURE CITED

ALMQUIST, H. J., AND E. L. R. STOKSTAD 1937 The gizzard factor of the chick. J. Nutrition, vol. 13, p. 339.

BIRD, H. R., O. L. KLINE, C. A. ELVEHJEM, E. B. HART AND J. G. HALPIN 1936 The distribution and properties of the anti-gizzard erosion factor required by chicks J. Nutrition, vol. 12, p. 571.

BIRD, H. R., AND J. J. OLESON 1938 Effectiveness of chondroitin as the anti-gizzard-erosion factor required by chicks. Proc. Am. Soc. Biol. Chem., J. Biol. Chem., vol. 123, p. xi.

CRANDALL, L. A., JR., G. M. ROBERTS AND J. W. GIBBS 1932 The beneficial effect of mucin and chondroitin in experimental liver damage. Proc. Soc. Exp. Biol. and Med., vol. 29, p. 1082.

CRANDALL, L. A., JR., G. M. ROBERTS AND L. D. SNORF 1936 The use of chondroitin in idiopathic headache (including migraine). Am. J. Digestive Dis. and Nut., vol. 3, p. 289.

SECONDARY DEFICIENCY OF VITAMIN B_1 AND RIBOFLAVIN IN THE BLACKTONGUE PRODUCING DIET [1]

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FOUR FIGURES

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In a previous study dealing with the optimum and minimum dose of nicotinic acid, Margolis, Margolis and Smith ('38) observed that dogs with experimental blacktongue failed to make satisfactory weight gains with treatment after the first and second consecutive attacks. The present study was designed to determine whether the gradual failure of nicotinic acid was due to an accumulated toxic effect or occurred as a result of secondary limiting factors in the diet.

METHOD

Experimental canine blacktongue was produced in adult dogs of mixed breed and both sexes, with an average weight of 16 kg., by feeding them Smith, Persons and Harvey's ('37) modification of Goldberger's diet no. 123 (table 1).

After the development of blacktongue, nicotinic acid was given daily for 10 days and then withdrawn. By this method, standardized by Harvey, Smith, Persons and Burns ('38), one can determine the immediate response to nicotinic acid, as well as the ability to store the active principle. The nicotinic acid, in a concentration of 5 mg. per milliliter, was dissolved

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[1] Part of the expenses of this work was covered by grants from the Rockefeller Foundation and the Lederle Biological Laboratories.

in water for oral treatment and in normal physiological saline (0.85%) for intramuscular and subcutaneous injections. Daily doses of 0.5 mg. to 10 mg. per kilogram of body weight were given, each dog receiving the same dosage throughout the 10-day period. The previous results of Margolis, Margolis and Smith ('38) indicated that all doses within this range produced the optimal curative effects for the first attack. The supplements of thiamin (Merck) and riboflavin (Merck and S. M. A.) were given orally.

TABLE 1

Composition of blacktongue-producing diet used

COMPONENTS	QUANTITY APPROXIMATE PER CENT	TOTAL GRAMS[1]	PROTEIN GRAMS	FAT GRAMS	CARBOHYDRATE GRAMS	VIT. B_1[4] I.U.
Cornmeal, yellow[2]	65	320.0	26.9	15.0	236.8	15–180
Cow peas (Vigna sinensis)	8	40.0	8.5	0.6	24.3	131
Casein,[3] purified	10	48.0	41.6			
Sucrose	5	25.5			25.5	
Cottonseed oil	5	24.0		24.0		
Cod liver oil	5	24.0		24.0		
Salt mixture[5]	2	9.5				
Total	100	491.0	77.0	63.6	286.6	146
Calories			308.0	572.4	1146.4	

[1] Amounts fed daily per dog. Total calories 2027.

[2] Cornmeal, cow peas (coarsely ground) and salt mixture are stirred into water and cooked in an aluminum steam cooker for 1½ hours. Then the other ingredients are poured in and mixed well.

[3] Commercial casein was leached with acidulated water and extracted with 95% alcohol. Details given by Harvey, Smith, Persons and Burns ('38).

[4] Vitamin B_1 requirement for the 16 kg. dog according to Cowgill's formula is 38.61 I.U.

[5] Underhill and Mendel ('28).

RESULTS

A study of nicotinic acid treatment of forty-five attacks of blacktongue in fourteen dogs revealed a decreasing ability of the animals to regain weight after consecutive attacks (table 2, fig. 1). There was also an apparent decrease in the ability of the dog to store the protective factor. It should be emphasized that the condition of the mouth was not so severe before the third and fourth treatments as it was before the first and second. Treatment became imperative at this point because of the precipitous weight loss.

These results were unexpected since a liver extract[2] was effective in producing repeated satisfactory remissions in

TABLE 2

A total of fourteen dogs on the blacktongue-producing diet treated with nicotinic acid in forty-five repeated attacks (one to four attacks per dog)

CONSECUTIVE ATTACKS	NUMBER OF ATTACKS	AVERAGE WEIGHT GAIN AFTER TREATMENT WITH NICOTINIC ACID	AVERAGE TIME OF RELAPSE AFTER NICOTINIC ACID WAS DISCONTINUED
			days
1st	14	1.58	28
2nd	14	1.40	20
3rd	10	0.76	13
4th	7	0.55	5

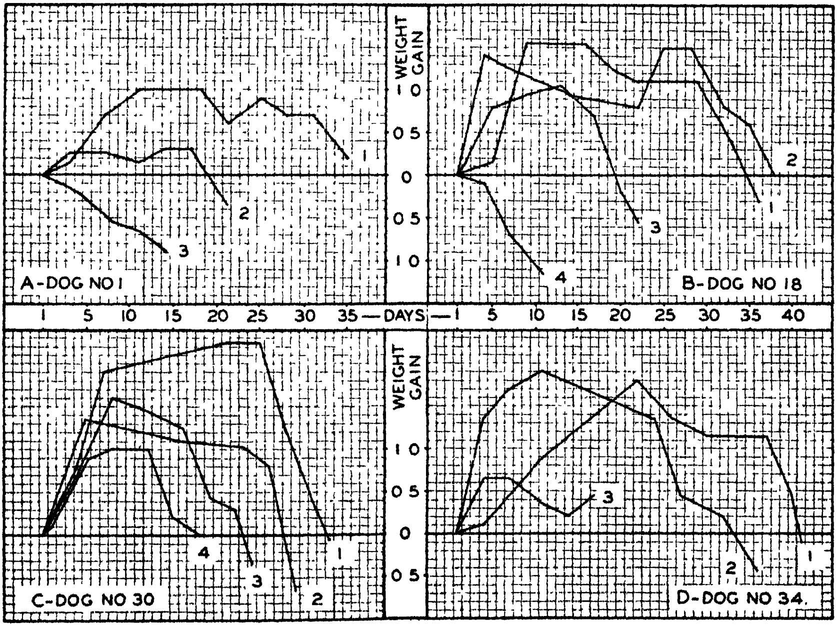

Fig. 1 (A, B, C, D) Weight responses in four dogs on consecutive treatments with nicotinic acid. (The order of treatments is indicated numerically.)

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[2] Oral residue and intramuscular extract. Kindly supplied by Lederle Laboratories, Pearl River, New York. This extract was shown by Ruffin and Smith ('37) to be curative of pellagra.

many consecutive attacks of blacktongue. Eleven dogs in sixty-eight attacks of blacktongue showed no decrease of weight response or storage capacity with liver treatment (table 3). Two dogs, after complete failure to respond to nicotinic acid, recovered promptly when given liver and were maintained on liver extract for 3 and 4 months, respectively. After withdrawal of the liver, both dogs relapsed with classical symptoms of blacktongue. One of these dogs then gave an excellent response to nicotinic acid (fig. 3 a), but the other received inadequate amounts and died.

TABLE 3

A total of eleven dogs on the blacktongue-producing diet treated with liver extract in a total of sixty-eight repeated attacks (one to ten attacks per dog)

CONSECUTIVE ATTACKS	NUMBER OF ATTACKS [1]	AVERAGE WEIGHT GAIN AFTER TREATMENT WITH LIVER EXTRACT	AVERAGE TIME OF RELAPSE AFTER LIVER WAS DISCONTINUED
		kg.	*days*
1st	11	1.41	23
2nd	11	1.61	24
3rd	11	1.75	21
4th	9	2.19	20
5th	9	1.49	22
6th	7	2.31	19
7th	4	1.78	14
8th	3	1.77	18
9th	2	1.43	25
10th	1	2.10	

[1] These dogs were in a series reported previously from this laboratory by Harvey, Smith, Persons and Burns ('38).

In view of these findings it was important to determine what factors present in liver in addition to nicotinic acid were responsible for these results. To achieve this, the pure substances associated with the vitamin G complex were fed separately as supplements to the basal diet.

1. Vitamin G (B_2) (riboflavin). In none of the four dogs treated with riboflavin was there any noticeable weight gain or increase in appetite. However, if riboflavin was omitted from the diet entirely or fed in insufficient amounts, there was collapse and finally death, often with total absence of mouth symptoms. The liver of the one dog examined at necropsy was found to be yellow and to contain large amounts of fat as revealed by section. Both of these findings confirm previous work of Sebrell et al. ('37, '38).

Two dogs, failing with nicotinic acid treatment, were given a supplement of riboflavin alone. The weight decrease continued and there was no change in appetite. Two other dogs, one of whom received no riboflavin and the other only 2 mg. as a supplement during the experimental period of 145 days, responded in their fourth attack of blacktongue with entirely adequate gains in weight and appetite when nicotinic acid was given, but died in collapse 7 and 10 days after the drug was discontinued. In both cases collapse occurred at the peak of the weight curve (fig. 2, a, b). One of these dogs had received adequate amounts of thiamin.

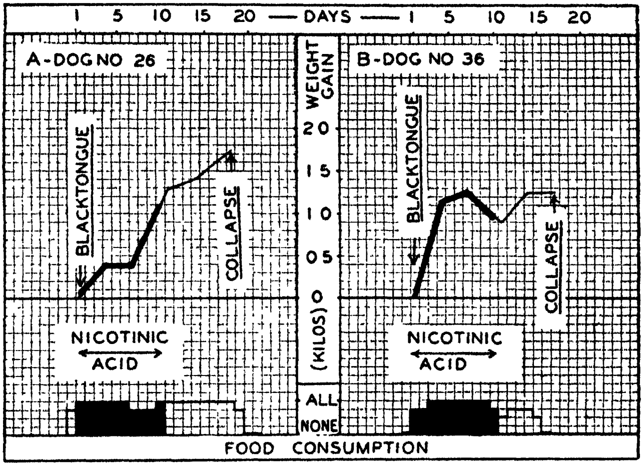

Fig. 2 (A, B) Weight and appetite responses to treatment with nicotinic acid of two dogs with blacktongue complicated by a riboflavin deficiency.

In the riboflavin deficient dogs we also observed a very characteristic type of dermatitis involving loss of hair and a scaly condition of the skin. This condition seemed to parallel the degree of riboflavin deficiency, yielding gradually to treatment with riboflavin. The condition is distinguished from mange by failing to heal with mange cure. Goldberger and Wheeler ('28) noted changes in the skin of the scrotum of their dogs with blacktongue, and Sebrell and Onstott ('38) noted a dermatitis quite similar to the type reported here in their riboflavin deficient dogs. Adams ('36) and Smith ('38) have also observed skin changes in vitamin G deficiency.

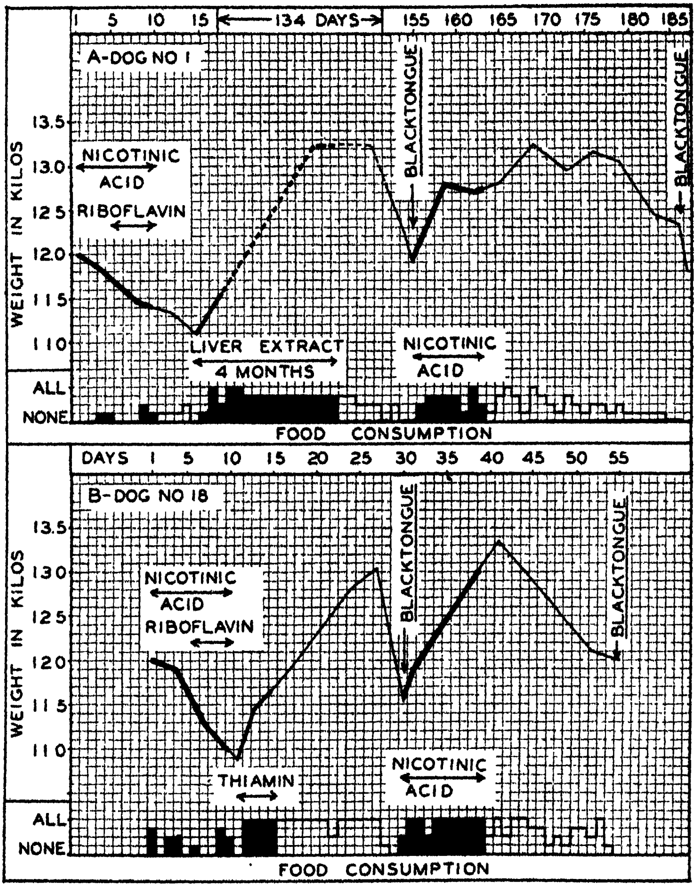

Fig. 3 (A). Weight response with nicotinic acid treatment of the third attack of blacktongue (fig. 1 a, curve 3), continued weight loss with addition of riboflavin, restoration with liver extract and subsequent weight response to nicotinic acid. (B). Weight response with nicotinic acid treatment of the fourth attack of blacktongue (fig. 1 b, curve 4), continued weight loss with addition of riboflavin, restoration with vitamin B_1 (thiamin), and subsequent weight response to nicotinic acid.

2. *Vitamin B_1* (thiamin chloride, Merck). No neurological symptoms of vitamin B_1 deficiency were observed during these experiments. It was possible to maintain one dog in excellent health for 11 months on the basal diet supplemented by nicotinic acid and riboflavin. But after one or two successive attacks of blacktongue, dogs began to show loss of appetite, inadequate weight gains, and finally falling weight curves in the absence of mouth symptoms. These symptoms were not altered by the administration of nicotinic acid or riboflavin. Four dogs were treated with vitamin B_1 (thiamin) with the following results: One dog responded with a 2.15 kg. gain in weight after complete failure to respond to nicotinic acid; two dogs, after showing a decreased response to nicotinic acid, improved with B_1, gaining 1.95 and 1.85 kg. respectively. A fourth dog which was still responding well to nicotinic acid had, at the end of the customary depletion period, a mouth that was only slightly suspicious of blacktongue [3] but had loss of weight and appetite. When 15 mg. of B_1 was fed, the weight and appetite increased promptly while the mouth lesions were advancing to a picture of severe blacktongue (fig. 4 a). The increased demand for vitamin B_1 with repeated attacks of blacktongue had no relation to the amount or severity of diarrhea in these dogs.

3. *Adenylic acid* (from muscle). Adenylic acid, 5 mg. fed daily for 10 days, when added to the diet after failure of nicotinic acid had no perceptible effect on the two dogs tested.

4. *Nicotinic acid, riboflavin and thiamin.* Seven dogs on the blacktongue producing diet were tested over a period of 7 to 13 months by supplementing the diet with riboflavin, thiamin and nicotinic acid. At the end of the experimental period all of these dogs were in excellent condition, at approximately their original weight, and free from macrocytic anemia (table 4).

Three of these dogs were maintained for 11 to 12 months by continuous administration of daily doses of 1 mg. thiamin and 1 mg. of nicotinic acid per kilogram of body weight and weekly doses of 1 to 2 mg. of riboflavin. At the end of this period there was no evidence of cumulative toxic effect from the nicotinic acid.

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[3] We have found along with other investigators that this mild condition of slightly reddened buccal mucosa has a tendency to come and go

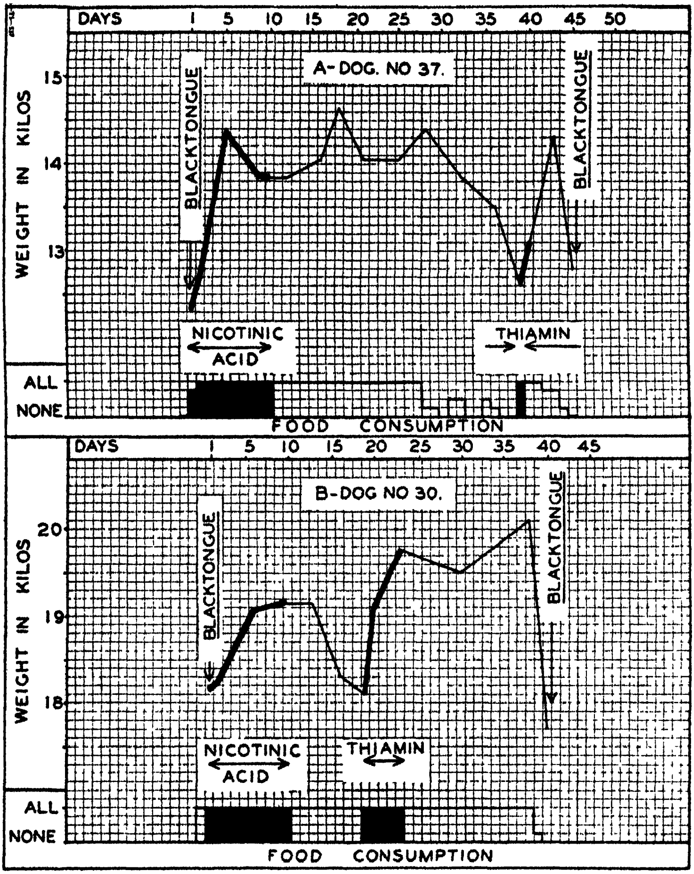

Fig. 4 (A). Anorexia and weight loss in the absence of blacktongue after second treatment with nicotinic acid, and subsequent weight and appetite response to vitamin B_1 (thiamin), while the mouth symptoms progressed to severe blacktongue. (B). Weight response in fourth treatment with nicotinic acid (fig. 1 c, curve 4), and subsequent weight gain with vitamin B_1 (thiamin). This curve demonstrates adequate storage of nicotinic acid in vitamin B_1 deficiency.

In figures 2, 3 and 4 food consumption is indicated by the four squares at the bottom of the chart, each square representing one-fourth of the daily ration. During treatment periods the food consumption is indicated in black. This period is also indicated by arrows on chart and by the heavier line in the weight curve.

Four other dogs were maintained on the basal diet supplemented at intervals with riboflavin and thiamin in amounts sufficient to prevent deficiency from these factors. Under these conditions twenty attacks of blacktongue, identical in every respect with the first attack on the unsupplemented diet, developed at regular intervals. The standard nicotinic acid treatment of these attacks gave uniformly satisfactory results comparable to those obtained with liver (table 5). One of

TABLE 4

Dogs maintained on the blacktongue-producing diet supplemented with riboflavin, thiamin and nicotinic acid

DOG NO.	PERIOD OF TREATMENT	HGB.	R.B.C.
Dogs given B_1 and riboflavin and nicotinic acid regularly			
	months	*gm.*	
30	12	14.9	6,005,000
32	11	14.2	6,130,000
33	11	9.2	4,900,000
34[1]	11	16.2	7,300,000
Dogs given B_1 and riboflavin regularly, nicotinic acid only in attacks of blacktongue			
18	13	15.4	7,500,000
37	9	13.0	5,445,000
49	7	12.6	5,300,000
51	7	13.0	5,500,000

[1] This dog did not receive vitamin B_1 (thiamin).

TABLE 5

A total of four dogs on the blacktongue-producing diet supplemented with thiamin and riboflavin treated with nicotinic acid in twenty repeated attacks (one to six attacks per dog)

CONSECUTIVE ATTACKS	NUMBER OF ATTACKS	AVERAGE WEIGHT GAIN AFTER TREATMENT WITH NICOTINIC ACID	AVERAGE TIME OF RELAPSE AFTER NICOTINIC ACID WAS DISCONTINUED
		kg.	*days*
1st	4	2.09	29
2nd	4	1.63	24
3rd	4	2.20	29
4th	4	2.06	20
5th	2	2.25	25
6th	2	2.58	

these dogs, after failing to respond to repeated treatments with nicotinic acid (fig. 1 c) while on the unsupplemented diet, gave satisfactory results with nicotinic acid in six consecutive attacks of blacktongue after the diet was supplemented with riboflavin and thiamin.

DISCUSSION

It is evident from the data submitted that the blacktongue producing diet used in these experiments is deficient in riboflavin. This deficiency has been demonstrated in the rat by Booher and Hansmann ('36) and Helmer and Fouts ('38) and in the dog by Zimmerman et al. ('34), Cowgill et al. ('37) and Sebrell et al. ('37 a, b, '38). We were able to confirm the finding of these investigators that in the absence of riboflavin, dogs die in collapse. It should be emphasized, however, that although the blacktongue diet is deficient in riboflavin, a factor undoubtedly required by the dog, it is not significant in the etiology of blacktongue. It has no curative effect on acute blacktongue, while on the other hand, dogs responding to nicotinic acid with prompt cure of the symptoms, often die suddenly in collapse due to riboflavin deficiency (fig. 2).

Helmer and Fouts ('38) observed that a powdered liver extract completely supplements the blacktongue producing diet when rats serve as the experimental animal. The present study shows that for the dog, liver extract also completely supplements the blacktongue producing diet, and further, that the combination of nicotinic acid, riboflavin and vitamin B_1 will replace the liver.

Although it was possible for us to maintain one dog in excellent condition for 11 months on the basal diet plus nicotinic acid and riboflavin, a vitamin B_1 deficiency was regularly produced in dogs suffering repeated attacks of blacktongue (figs. 1, 3 and 4). These attacks, so like pellagra in their periodic recurrences, gastro-intestinal lesions, sore mouth, infection and metabolic disturbance, obviously led to an increased demand of the dog for vitamin B_1. This greater requirement could not be correlated with food consumption[4] or diarrhea[5] which was never of sufficient duration or severity in our experiments to play an important part.

In the presence of vitamin B_1 deficiency the specific effect of nicotinic acid on the mouth symptoms was not altered nor was the storage period of this factor decreased. The apparent interference with storage which is indicated in table 2 was due to complete nutritional failure which necessitated treatment before the mouth symptoms were severe. At this early stage of the experiment no other treatment than nicotinic acid was considered. It was subsequently found that for normal response to nicotinic acid with prompt gain in weight and appetite an adequate supply of vitamin B_1 is essential.

Since the natural blacktongue producing diet, on which the experimental ones are based, and the natural pellagra producing diet as found in the endemic areas of the South are almost identical it is not surprising to find peripheral neuritis occasionally associated with pellagra (Sydenstricker and Armstrong, '37). The present study indicates that while vitamin B_1 is deficient in the diet and required for the well being of the dog it is not etiologically significant in the production of blacktongue, the analogue of human pellagra.

In patients with endemic pellagra peripheral neuritis has never been considered part of the syndrome, whereas it is a significant and diagnostic part of the beri beri syndrome (Eddy and Dalldorf, '37). It is well known that vitamin deficiencies in the human are complicated and rarely, if ever, limited to one factor. Where peripheral neuritis occurs in association with pellagra, the neuritic symptoms may be cured by vitamin B_1, while the pellagra symptoms grow worse (Spies and Aring, '38; Smith and Ruffin, '38).

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[4] According to Cowgill's formula the daily requirement of vitamin B_1 for the 16 kg. dog is 38.61 I.U. The portion of the diet fed supplies in the uncooked state 146 to 311 I.U. of the vitamin (Fixsen and Roscoe, '38). The exact amount of destruction of vitamin B_1 by cooking the natural carriers (cornmeal and cow peas) in the presence of the salt mixture is not known.

[5] Dann and Cowgill ('38) found that the vitamin B_1 requirement for the dog was increased by an artificially induced diarrhea.

It is to be expected, then, that vitamin B factors with their various combinations in natural foodstuffs will give rise to deficiencies characterized by different combinations of symptoms. The primary or predominant deficiency determines the character of the syndrome which results, although there may be various accompanying symptoms attributable to other minor deficiencies.

SUMMARY AND CONCLUSIONS

Thirty-three dogs in 133 attacks of blacktongue were studied in their response to treatment with 1) liver, 2) nicotinic acid alone, 3) adenylic acid from muscle, and 4) nicotinic acid combined with vitamin B_1 (thiamin) and vitamin B_2 (riboflavin).

1. A study of sixty-eight attacks of blacktongue in eleven dogs indicated that liver extract completely supplements the blacktongue producing diet under the conditions of our experiments.

2. A study of forty-five attacks of blacktongue in fourteen dogs revealed the inability of nicotinic acid to replace liver as a complete supplement to the diet, although in the first and often in the second attack the results with nicotinic acid were comparable to those with liver.

3. Two dogs were treated with adenylic acid but results were not conclusive.

4. Twenty attacks of blacktongue in four dogs maintained on the basal diet supplemented with vitamin B_1 (thiamin) and vitamin B_2 (riboflavin) when treated with nicotinic acid gave results that were in every way comparable with the results obtained with liver. Three dogs were maintained on the basal diet supplemented with these three factors for 11 to 12 months in normal condition. There was no cumulative toxic effect from the nicotinic acid in these three dogs, nor was there any macrocytic anemia in any of the seven dogs.

It is concluded then that under the conditions of these experiments both riboflavin and vitamin B_1 (thiamin) are secondary deficiencies of our blacktongue producing diet. It appears also that the dog's requirement for vitamin B_1 is markedly increased by repeated attacks of blacktongue.

LITERATURE CITED

ADAMS, P. D. 1936 The oxygen uptake and composition of the skin of rats in vitamin G deficiency. J. Biol. Chem., vol. 116, p. 641.

BOOHER, L. E., AND G. H. HANSMANN 1935 Vitamin G concentrates as preventives against blacktongue. Am. J. Physiol., vol. 114, p. 429.

COWGILL, G. R., H. M. ZIMMERMAN AND E. BURACK 1934 Studies of vitamin G (B_2) deficiency in dogs: preliminary communication. Am. J. Physiol., vol. 109, p. 24.

DANN, M., AND G. R. COWGILL 1938 Influence of diarrhea on the vitamin B requirement. Arch. Int. Med., vol. 62, p. 137.

EDDY, W. H., AND G. DALLDORF 1937 The avitaminoses. The Williams and Wilkins Co., Baltimore, Md., pp. 75, 92.

FIXSEN, M. A. B., AND M. H. ROSCOE 1938 Tables of the vitamin content of human and animal foods. Nutr. Abs. and Rev., vol. 7, p. 823.

GOLDBERGER, J., AND G. A. WHEELER 1928 Experimental black tongue of dogs and its relation to pellagra. Pub. Health Rep., vol. 43, p. 172.

HARVEY, H. I., D. T. SMITH, E. L. PERSONS AND M. V. BURNS 1938 The effect of various fractions of liver on experimental canine blacktongue. J. Nutrition, vol. 16, p. 153.

HELMER, O. M., AND P. J. FOUTS 1938 Multiple nature of the deficiency of the blacktongue producing diets as shown by studies on rats. J. Nutrition, vol. 16, p. 271.

MARGOLIS, G., L. H. MARGOLIS AND S. G. SMITH 1938 The cure of experimental canine blacktongue with optimal and minimal doses of nicotinic acid. J. Nutrition, vol. 16, p. 541.

RUFFIN, J. M., AND D. T. SMITH 1937 A clinical evaluation of the potency of various extracts of liver in the treatment of pellagra. South. Med. J., vol. 30, p. 4.

SEBRELL, W. H., R. H. ONSTOTT AND D. J. HUNT 1937 The treatment of blacktongue with a preparation containing the 'filtrate factor,' and evidence of riboflavin deficiency in dogs. Pub. Health Rep., vol. 52, p. 427.

SEBRELL, W. H., J. D. HUNT AND R. H. ONSTOTT 1937 Lactoflavin in the treatment of canine blacktongue. Pub. Health Rep., vol. 52, p. 235.

SEBRELL, W. H., AND R. H. ONSTOTT 1938 Riboflavin deficiency in dogs. Pub. Health Rep., vol. 53, p. 83.

SMITH, D. T., E. L. PERSONS AND H. I. HARVEY 1937 On the identity of the Goldberger and Underhill types of canine blacktongue. Secondary fuso-spirochetal infection in each. J. Nutrition, vol. 14, p. 373.

SMITH, D. T., AND J. M. RUFFIN 1938 Personal communication.

SMITH, S. G. 1938 Etiology of sebaceous gland atrophy in the rat in avitaminosis. J. Nutrition, vol. 15, p. 45.

SMITH, S. G., G. MARGOLIS AND L. H. MARGOLIS 1938 Effect of autoclaving on the vitamin potency of nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 251.

SPIES, T. D., AND C. D. ARING 1938 The effect of vitamin B_1 on the peripheral neuritis of pellagra. J. Am. Med. Assoc., vol. 110, p. 1081.

SYDENSTRICKER, V. P., AND E. S. ARMSTRONG 1937 A review of four hundred and forty cases of pellagra. Arch. Int. Med., vol. 59, p. 883.

UNDERHILL, F. P., AND L. B. MENDEL 1928 A dietary deficiency canine disease—further experiments on the diseased condition in dogs described as pellagra-like by Chittenden and Underhill and possibly related to so-called black tongue. Am. J. Physiol., vol. 83, p. 589.

ZIMMERMAN, H. M., G. R. COWGILL AND J. C. FOX 1937 Neurologic manifestations in vitamin G (B_2) deficiency. Arch. Neurol. and Psychiat., vol. 37, p. 286.

ON THE DIGESTIBILITY OF RAW POTATO STARCH IN MAN

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In 1920 the previously debated question as to whether raw starch is utilizable by man was apparently settled in the affirmative by the observations of Langworthy and Deuel ('20). They found that relatively large quantities (426 to 773 gm.) of raw corn or wheat starch could be fed to normal human subjects without any appearing in the feces. Raw potato starch proved to be less 'digestible' than the other two, but, even so, they were able to recover in the feces only 5 to 38% of that (468 to 710 gm.) ingested. These observations appear to be largely responsible for the fact that it is now sometimes stated in textbooks that large quantities of raw starch can be digested and assimilated by man.

During the course of various in vitro experiments, we were forced to the conclusion that raw potato starch is almost entirely refractory to the action of gastro-intestinal enzymes. Since this was apparently inconsistent with the observations of Langworthy and Deuel ('20), we have repeated and extended their study on the digestion of raw potato starch by normal human subjects.

METHODS

In vitro experiments. Pancreatin was used in conducting the in vitro experiments since it offered a reproducible source of highly active enzymes. The preparation employed throughout the study possessed the following activity: trypsin, double

U. S. P. standard; lipase, as determined by the method described by Cherry and Crandall ('32), 4400 units per gram; diastase, as determined by Willstater's method (Schmidt, Greengard and Ivy, '34), 7 units per gram.

The quantity of reducing substance produced by incubating raw potato starch with an excess of pancreatin was compared with that produced by comparable treatment of the same quantity of boiled starch. Each determination was controlled by determining the reducing substances formed from the pancreatin alone and from the raw starch alone. Thus, by considering the quantity of reducing substance produced by the digestion of the boiled starch as representing total possible digestion, it was possible to calculate the percentage digestion of the raw starch. The proceduce in detail was as follows.

Four 125-cc. Erlenmeyer flasks were prepared as is shown in the following tabulation:

Flask no.	*Quantity of starch (mg.)*	*Treatment of starch*	*Quantity of pancreatin (cc. 2% sol.)*	*Quantity of buffer (pH 6.9) (cc.)*
1	500	Raw	5	25
2	500	Boiled	5	25
3	500	Raw	0	30
4	0	......	5	25

The flasks were incubated at 37°C., with sufficient aggitation to keep the starch in suspension, for periods ranging from 3 to 20 hours. At the end of the incubation period the material was filtered and the reducing sugar concentration of the filtrates was determined by the hypoiodate titration method (Schmidt, Greengard and Ivy, '34). The total quantity of reducing substance found in flasks 3 and 4 was subtracted from that found in flasks 1 and 2 respectively, leaving the quantity of reducing substance produced by the enzymatic digestion of the starch.

In vivo experiments: ten healthy medical students were used as subjects for the experiment. The composition of the starch diet, which was essentially the same as that used by Langworthy and Deuel ('20), was milk 63%, raw potato starch 21%, peanut oil 8.7% and sucrose 7.3%.

During the first test period on five of the subjects the diet was prepared in the form of a frozen pudding, thus reduplicating the experimental conditions of Langworthy and Deuel. During subsequent tests the diet was consumed as an unfrozen fluid. In all cases a readjustment period of 1 month was permitted between consecutive tests on any given subject.

The tests were conducted in the following manner. The subjects were given 1281 gm. of the starch diet per day for a 3-day period (nine meals). Thus, during each 3-day period a total of 807 gm. of starch was ingested. During this period they were permitted to drink tea, coffee and fruit juices as desired, but no other food. A carmine 'marker' was given with the first meal of the period and again with the last. All feces were collected between the first appearance of the first dose of carmine and the complete disappearance of the second dose. The starch content of the feces was determined by a method which we have previously described (Beazell, Schmidt and Ivy, '37).

The first phase of the study was concerned with the determination of the effect of freezing on the digestibility of the raw starch. Five of the subjects were given the diet in the form of a frozen pudding and the fraction of the ingested starch lost in the feces was determined. Subsequently the test was repeated except that the diet was unfrozen. In the second phase of the study the other five subjects were employed to determine the effect of large doses of a potent diastatic enzyme on the utilization of the raw starch. The starch loss was determined first, during a control period, and second, during a period in which 8 gm. of 'takadiastase' were taken with each meal. 'Takadiastase' was chosen for the study because it is a potent source of diastatic enzyme, and because it is inactivated at a relatively low pH. This latter quality permits the major portion of that ingested to survive the gastric acidity (Ivy, Schmidt and Beazell, '36).

RESULTS

In vitro experiments. The results of a number of these experiments are shown in table 1. It will be seen that the quantity of reducing sugar obtained from the digestion of the raw starch never exceeded 7.6% of that obtained from the digestion of the boiled starch. Furthermore, lengthening the incubation period to 20 hours did not increase the quantity of raw starch digested. This latter fact suggests that a constant fraction of the raw starch may have been more easily digested than the remainder, perhaps as a result of mechanical rupture of the envelope of the starch granule during milling.

TABLE 1

In vitro digestion of potato starch

(500 mg. starch incubated with 100 mg. pancreatin)

TRIAL	INCUBATION PERIOD	REDUCING SUGAR (NET)[1]		PER CENT DIGESTION OF RAW STARCH
		From boiled starch	From raw starch	
	hours			
1	3½	367	28.0	7.6
2	5	418	19.8	4.7
3	20	500	23.0	4.6

[1] Expressed as milligrams maltose.

In vivo experiments. The effect of freezing the starch on the quantity lost in the feces is clearly demonstrated by the results recorded in table 2. During the period when the subjects ingested the starch meal in the form of a frozen pudding, the average starch loss of the five subjects was 26%. Thus, freezing caused less starch to be lost in the feces.

The addition of the 'takadiastase' to the diet had no effect on the quantity of starch lost in the feces. The results of this phase of the study are recorded in table 3. It will be seen that the differences between the two periods are all attributable to spontaneous variations.

Flatulence usually accompanied by cramp-like pain was a prominent and distressing symptom in all of the subjects. In some cases it was sufficiently severe (++++ flatulence) to be incapacitating. Since it is obviously impractical to make quantitative gas collections in a study of this nature, it was necessary to evaluate the degree of flatulence on the bases of the subjective sensations of the subjects. Thus, any attempt to draw conclusions from minor changes in the reported degree of flatulence would be hazardous. However, the first group of subjects uniformly reported that they were much less flatulent while ingesting the unfrozen meal than they had

TABLE 2

Effect of freezing on the quantity of ingested raw starch excreted in the feces (values are for entire metabolic period)

SUBJECT	STARCH DIET FROZEN					STARCH DIET UNFROZEN				
	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence
	gm.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*		
J.B.	933	311	195.4	24.2	++++	1439	417	310	38.2	+++
J.J.	944	262	191.6	23.6	+++	1514	486	342	42.2	+
R.M.	1203	400	317.8	39.2	+++	1575	512	426	53.0	++
H.G.	562	225	195.4	24.2	++	1383	458	344	42.5	+
R.B.	635	160	149.4	18.5	++++	953	292	227	28.0	+++
Av.	855	271	209.9	26.0		1373	433	329.8	40.9	

TABLE 3

Effect of orally administered diastase on the quantity of ingested raw starch excreted in the feces (values are for entire metabolic period)

SUBJECT	CONTROL PERIOD (DIET UNFROZEN)					ENZYME PERIOD (DIET UNFROZEN)				
	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence	Wet weight feces	Dry weight feces	Total starch in feces	Per cent ingested starch excreted	Flatulence
	gm.	*gm.*	*gm.*			*gm.*	*gm.*	*gm.*		
R.R.	1356	309	130.6	15.4	++++	1723	420	172.8	21.4	+++
R.C.	1306	522	363.0	41.6	+++	1457	456	289.0	35.6	+++
J.S.	1462	573	415.4	49.0	++	1606	596	446.0	55.0	+++
W.V.	1851	662	462.7	57.3	+++	2047	719	461.3	57.2	+++
W.B.	1670	480	290.4	36.0	+	1563	466	283.0	35.0	+++
Av.	1529	509	330.4	39.7		1679	527	330.4	40.8	

been while ingesting the frozen meal. In every case the change was so marked that there was little room for questioning its significance.

DISCUSSION

Langworthy and Deuel ('20) conducted a series of seven experiments on five subjects in their investigation of the digestibility of raw potato starch. They employed the frozen pudding in all of their tests. The average starch loss in their series was 21.8% (4.8 to 37.7%). The slight differences between this value and that obtained by us, when the frozen pudding was employed (26.0%) can be attributed to individual variation.

The results of the first phase of the in vivo experiments establishes beyond any reasonable doubt that freezing does modify the quantity of starch lost in the feces. In the case of each of the five subjects the quantity lost was significantly greater during the period in which the unfrozen meal was being ingested. The differences are all too marked to be attributed to spontaneous individual variations. Justification for this latter statement can be found in the uniformity of results between the two periods in the subsequent experiment in which the unfrozen diet was ingested during both periods (table 3).

It should be noted that throughout this paper the term 'starch loss' has been employed rather than 'starch utilization' or 'starch digestion.' The latter two terms imply useful digestion; the term 'starch loss' is noncommittal. When all of the facts are correlated, it appears evident that bacterial fermentation accounts for a significant fraction of the raw starch that disappears during passage through the human gastro-intestinal tract. In the first place, the uniform extreme flatulence establishes active fermentation, and the fact that flatulence was greatest when the starch loss was least (frozen pudding period) suggests that fermentation played an important part in the increased break down of the starch.

In the second place, the failure of large doses of potent diastase to affect the starch lost in the feces, indicates that amylolytic enzymes are of minimal importance. Finally, it is difficult to reconcile the results of the in vivo and in vitro experiments unless bacterial fermentation is taken into account.

CONCLUSIONS

1. The significance of values for the 'digestibility' of raw starch is open to question when they have been obtained by feeding the starch in the form of a frozen pudding.
2. The fact that a given quantity of raw starch disappears during passage through the gastro-intestinal tract does not mean that all that has disappeared has been usefully digested and assimilated. Indeed, the evidence suggests that in the case of raw potato starch, bacterial fermentation accounts for an important part of the starch that is decomposed during passage through the alimentary tract.

LITERATURE CITED

BEAZELL, J. M., C. R. SCHMIDT AND A. C. IVY 1937 On the effectiveness of orally administered diastase in achylia pancreatica (dog). J. Nutrition, vol. 13, p. 29.

CHERRY, I. S., AND L. A. CRANDALL, JR. 1932 The specificity of pancreatic lipase. Am. J. Physiol., vol. 100, p. 266.

IVY, A. C., C. R. SCHMIDT AND J. M. BEAZELL 1936 On the effectiveness of malt amylase on the gastric digestion of starches. J. Nutrition, vol. 12, p. 59.

LANGWORTHY, C. F., AND H. J. DEUEL 1920 Digestibility of raw corn, potato and wheat starches. J. Biol. Chem., vol. 42, p. 270.

SCHMIDT, C. R., H. GREENGARD AND A. C. IVY 1934 A comparison of methods for the quantitative estimation of diastase in duodenal fluid. Am. J. Dig. Dis. and Nutr., vol. 1, p. 618.

DIET AND RATE OF DEPLETION OF HEPATIC VITAMIN A[1,2]

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INTRODUCTION

It is generally recognized that the liver is the chief depot for vitamin A storage. Baumann, Riising and Steenbock ('34) showed that when these stores were drawn upon, the rate of depletion was directly proportional to the amount of vitamin A present. Davies and Moore ('35) and Ruiz ('35) reported that very large stores of vitamin A were rapidly depleted until a certain level was reached; after that the rate of depletion was much slower. Greaves and Schmidt ('36) found that choledochocolonostomized rats or rats given thyroxine were depleted more rapidly than normal rats. Thyroidectomized rats showed a decreased rate of depletion, while icteric, laparotomized and dinitrophenol-treated rats depleted their reserves at rates comparable to normal rats. The relationship of vitamin A and thyroxine has recently been reviewed by Murlin ('38). Green ('34) noted that young rats survived for approximately the same length of time on vitamin A-free diets which differed greatly in fat content.

In connection with out earlier studies (Lease, Lease, Weber and Steenbock, '38) on the effect of rancidity on vitamin A

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[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

[2] The authors wish to express their sincere appreciation to Lever Brothers Company whose financial support made the work possible.

destruction, the question arose as to whether rancid fats tended to destroy hepatic stores of vitamin A. It has been shown by several workers that rancid fats in a diet containing vitamin A or carotene tend to induce a vitamin A deficiency (Fridericia, '24; Powick, '25; Mattill, '27; Nelson, Nelson and Lowe, '34; Lease, Lease, Weber and Steenbock, '38). However, the effect of rancid fat on liver stores of vitamin A had not been investigated. The experiments now reported deal with the effect of high-, low- and rancid-fat rations on this aspect of the problem. The effect of speedy depletion of excess fat from the liver by the use of choline (Best and Huntsman, '35) on vitamin A reserves was also investigated.

METHODS

The rats were fed a basal ration composed of casein 18%, brewers' yeast 8%, salts (Osborne and Mendel, as modified by Hawk and Oser, '31) 5%, and sucrose 69%. When fat was included, it was added at the expense of an equivalent amount of sucrose. The fresh lard was a high quality, kettle-rendered product. The rancid lard was prepared by aerating the fresh lard with moist air at 50°C. in the presence of 1 mg. of copper acetate per 100 gm. of fat and with intense illumination from Mazda lamps. Halibut liver oil [3] was used as the source of vitamin A. The choline hydrochloride was incorporated in the ration after being dissolved in a small amount of water.

The vitamin A content of the livers was determined colorimetrically (Lease, Lease, Weber and Steenbock, '38), using the Carr and Price ('26) antimony trichloride reagent. The fat content of the livers was determined by titration of the liberated fatty acids of the fat as outlined by Hynd and Rotter ('30).

RESULTS AND DISCUSSION

Effect of rancid fat. Uniform reserves of vitamin A were produced in rats 4 weeks of age by giving 1500 blue units of vitamin A as halibut liver oil daily for 4 days. Two days later their livers were found to contain an average of 1048 blue units. These reserves were allowed to deplete on diets low in vitamin A but high, respectively, in fresh or rancid fat. After various periods of time the vitamin A content of the livers of a few animals from each group was determined. Analysis after 14, 28 and 56 days revealed no significant difference in the stores of rats which had been fed 30% of rancid lard or 30% of fresh lard (table 1). The rancid lard used in these experiments was only moderately rancid, having a peroxide number of 30.

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[3] The halibut liver oil was the commercial Haliver Oil furnished by Abbott Laboratories.

TABLE 1

Effect of fresh and rancid fat rations on hepatic stores of vitamin A [1]

WEEKS ON DIET	BLUE UNITS OF VITAMIN A PER LIVER		
	Initial	30% fresh lard	30% rancid lard per. no. 30
Initial	1048		
After 2 weeks		845	902
After 4 weeks		555	556
After 8 weeks		482	450

[1] Each value represents an average of four or more rats analyzed individually.

In another experiment, the effect of very rancid fat was determined in older animals. Adult males of similar weight, age and dietary history as taken from the stock colony were given fresh lard or very rancid lard (peroxide number 200) for a period of 40 days. To facilitate consumption of the ration its fat content was increased approximately 10% weekly, starting at a 30% level and ending with 67%. Analyses of the livers revealed similar rates of depletion irrespective of whether fresh or rancid fat rations had been fed.

Effect of high- and low-fat diets. In some of our experiments the fat content of the diets was high enough to produce fatty livers during the depletion period. It was possible that the amount of fat in the diet had some effect on vitamin A stores and their metabolism. The effect of dietary fat on the depletion of vitamin A previously stored was, therefore, investigated.

For this purpose rats 4 weeks of age were placed on the basal ration and given 1 drop of halibut liver oil (1500 blue units of vitamin A daily) for 5 days. Three days later an average of 2114 blue units of vitamin A per liver was found. Half of the animals were continued on the basal ration containing no additional fat; the other half were given the 30% fresh lard ration. Analyses of livers at intervals for 15 weeks (table 2), showed that the animals on the high-fat diet lost slightly more vitamin A than those on the low-fat ration. However, there was no clear-cut distinction between the two. In a second feeding trial extending over 8 weeks, entirely similar results were obtained.

TABLE 2

Effect of high and low fat rations on the rate of vitamin A depletion[1]

WEEKS ON DIET	BLUE UNITS VITAMIN A PER LIVER		
	Initial	Basal ration + 30% fresh lard	Basal ration
Initial	2114		
3 weeks		1974	2086
6 weeks		1344	1722
9 weeks		1162	1236
12 weeks		601	671
15 weeks		334	411

[1] Each value represents an average of three or more rats analyzed individually.

From these data it appears that the rate of depletion of moderately high reserves of vitamin A is not greatly affected by the amount of fat in the diet. These results are in line with those of Green ('34) who concluded that the more obvious steps of fat metabolism do not involve the use of vitamin A.

Influence of fat mobilization. Since vitamin A and its esters are fat-soluble, it seemed possible that the rapid depletion of fat from a fatty liver might increase the rate of unloading its vitamin A. To obtain information on this point, rats 3 weeks of age were given the basal ration containing 30% of fresh lard for a period of 40 days. On the first, fourteenth and twenty-eighth day of feeding they were given graded amounts of vitamin A as shown in table 3. On the fortieth day a few representative animals were killed for analysis. The remaining animals were divided into two groups; one was continued on the basal ration, while the other was given a supplement of 1.5% of choline. Analyses of the livers of representative animals revealed the rapid loss of fat on the choline diet, and none from the other; the vitamin A reserves were the same for both (table 3).

After the major part of our work had been completed, Thorbjarnson and Drummond ('38) reported that the rate of disappearance of vitamin A from the liver was greater with a low-fat diet than with a high-fat diet and that choline plus the high-fat diet tended to increase the rate of depletion. They used rats with very large stores of vitamin A. Our data are related to more physiological levels of storage. Thorbjarnson and Drummond were working with animals with enormous stores of vitamin A induced by feeding approximately 9000 blue units per day for 4 weeks. As Davies and Moore ('35) have shown, considerable variation exists in the amount of vitamin A contained in the livers of rats fed massive doses. They also showed that when liver stores are very high, very rapid depletion takes place over a short period of time, after which a much slower and more steady depletion is observed. Thorbjarnson and Drummond were studying the influence of fat and choline at this period of rapid and

TABLE 3

Effect of choline on the rate of depletion of vitamin A from fatty livers[1]

DAYS ON DIET	TOTAL BLUE UNITS OF VITAMIN A FED	ON BASAL RATION		ON BASAL RATION + 1.5% CHOLINE	
		Blue units vitamin A per liver	Per cent of fat in liver	Blue units vitamin A per liver	Per cent of fat in liver
0	3000	616	16.6		
4		612	16.3	598	9.8
12		585	16.6	564	1.5
20		545	17.0	539	1.7
0	4500	1072	14.6		
12		963	14.1	1111	5.7
0	9000	2409	13.6		
12		2035	15.9	2233	6.6

[1] Each figure represents an average of four or more livers analyzed individually.

variable depletion, which may well account for the divergence in results.

SUMMARY

The rate of loss of vitamin A from the liver of the rat was not affected greatly, if at all, by the amount of fat in the diet, by the feeding of rancid fats, nor by the rapid depletion of fat from the liver as effected by the administration of choline.

LITERATURE CITED

BAUMANN, C. A., B. M. RIISING AND H. STEENBOCK 1934 Fat-soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, p. 705.

BEST, C. H., AND M. E. HUNTSMAN 1935 The effect of choline on the liver fat of rats in various states of nutrition. J. Physiol., vol. 83, p. 255.

CARR, F. H., AND E. A. PRICE 1926 Color reactions attributed to vitamin A. Biochem. J., vol. 20, p. 497.

DAVIES, A. W., AND T. MOORE 1935 Vitamin A and carotene. XII. The elimination of vitamin A from the livers of rats previously given massive doses of vitamin A concentrate. Biochem. J., vol. 29, p. 147.

FRIDERICIA, L. S. 1924 Inactivating action of some fats on vitamin A in other fats. J. Biol. Chem., vol. 62, p. 471.

GREAVES, J. D., AND C. L. A. SCHMIDT 1936 Studies on the vitamin A requirements of the rat. Am. J. Physiol., vol. 116, p. 456.

GREEN, H. N. 1934 Fat metabolism in vitamin A deficiency: the utilisation of fat and the desaturation of fat in the liver. Biochem. J., vol. 28, p. 25.

HAWK, P. B., AND B. L. OSER 1931 A modification of the Osborne-Mendel salt mixture. Science, vol. 74, p. 369.

HYND, A., AND D. L. ROTTER 1930 Studies on the metabolism of animals on a carbohydrate-free diet. I. The distribution of glycogen and fat in the liver of animals fed on a carbohydrate-free diet. Biochem. J., vol. 24, p. 1390.

LEASE, E. J., JANE G. LEASE, JANET WEBER AND H. STEENBOCK 1938 Destruction of vitamin A by rancid rats. J. Nutrition, vol. 16, p. 571.

MATTILL, H. A. 1927 The oxidative destruction of vitamins A and E. J. Am. Med. Assoc., vol. 89, p. 1505.

MURLIN, JOHN R. 1938 Vitamins and hormones. J. Am. Diet. Assoc., vol. 14, p. 397.

NELSON, V. E., P. M. NELSON AND B. LOWE 1934 The effect of hydrogenated lard, storage lard and heated lard on the destruction of vitamin A in foods. Iowa Agric. Exp. Station Report, p. 81.

POWICK, W. C. 1925 Inactivation of vitamin A by rancid fat. J. Agric. Res., vol. 31, p. 1017.

RUIZ, A. S. 1935 Mechanism of elimination of vitamin A. Anales soc. españ. fís. quím., vol. 33, p. 820; Chem. Abstr., vol. 30, p. 3482 (1936).

THORBJARNSON, T., AND J. C. DRUMMOND 1938 Conditions influencing the storage of vitamin A in the liver. Biochem. J., vol. 32, p. 5.

THE BIOLOGICAL VALUE OF CAROTENE IN VARIOUS FATS [1, 2]

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INTRODUCTION

In the early days of vitamin A research, little attention was given to the nature of the fat in which the vitamin was administered. However, variations were soon observed in the growth response of rats when carotene or vitamin A was given in different solvents. Failure to demonstrate the vitamin A activity of carotene in ethyl oleate (Duliere, Morton and Drummond, '29) was attributed to the instability of carotene in this solvent (Hume and Smedley-MacLean, '30; Drummond, Ahmad and Morton, '30). Marked differences in the stability of vitamin A in various solvents were found by Dann ('34), and similar variations in the stability of carotene were demonstrated by Baumann and Steenbock ('33). The stability of carotene in ethyl oleate or ethyl laurate was found to be markedly increased by the use of antioxidants (Olcovich and Mattill, '31). When hydroquinone was added to carotene in an 'unstable solvent,' the carotene became stable, and vitamin A activity could be demonstrated. It appeared that the variations in biological results obtained with different solvents were due to the various degrees of instability of the active materials in these solvents.

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[2] The authors wish to express their appreciation to Lever Brothers Company whose financial support made this work possible.

More recently, however, it has been claimed that certain variations in biological response could not be attributed to the destruction of the active agent before administration. The evidence is reviewed in a report of the Medical Research Council ('35). Lathbury and Greenwood ('34) reported that different samples of arachis oil and coconut oil varied in their suitability as solvents for assaying vitamin A or carotene biologically and that quinol did not render an inferior oil suitable. Dyer, Key and Coward ('34) reported that "a solution of the standard (carotene) in arachis oil had five to six times the potency of solutions in hardened cottonseed oil and ethyl laurate, though made of the same strength . . . . Cod liver oil Z dissolved in coconut oil (161441) was about three times as active as when dissolved in hardened cottonseed oil or olive oil." De ('37) reported that large doses of carotene appeared to be better utilized when fed in red palm oil than in foods such as amaranth. Kraybill and Shrewsbury ('36) studying the growth response of rats fed carotene in cottonseed oil [3] and in decolorized butterfat observed that in order to obtain a given growth response, two to three times as much carotene was needed in treated butterfat as in cottonseed oil. They reported that carotene was stable in their samples of treated butterfat. Coward ('36) made comparisons of the variation in response of rats fed carotene dissolved in various oils, and concluded that there was no difference in the extent of the variations.

Variations in the potency of carotene dissolved in various oils have also been attributed to differences in absorption from the tract. The deleterious effect of mineral oil on carotene absorption is well known (Dutcher et al., '34; Jackson, '31, '34). In view of the large number of determinations of vitamin A and carotene made on foodstuffs by chemical methods, it is of practical importance to determine to what extent such analytical results are a true measure of biological potency. Obviously, if the potency of a milligram of carotene varies with the medium in which it is administered, chemical

[3] Wesson oil brand.

data can have only limited biological application. Kraybill and Shrewsbury ('36) have already intimated that chemical analyses are inadequate as a measure of the vitamin potency of butterfat. We have, therefore, studied the biological response of rats to carotene administered in various oils, using growth, cure of ophthalmia and the storage of vitamin A in the liver as biological criteria.

EXPERIMENTAL

Vitamin A storage. The effect of various fats on the storage of vitamin A in the liver was studied as follows: rats 3 to 4 weeks of age were placed on a ration consisting of casein 18, yeast 8, salts (Osborne and Mendel as modified by Hawk and Oser, '31) 4, dextrin 40, fat 30. The fats studied were decolorized butterfat, lard, soy bean oil, cottonseed oil,[4] and hardened cottonseed oil.[5]

The butterfat used had been decolorized as follows (Kraybill and Shrewsbury, '36): 20 gm. of Lloyd's reagent were added to 800 gm. of butterfat in a 2-liter flask at 50°C. and the mixture stirred periodically for 8 hours under nitrogen. The fat was filtered with suction through a wad of cotton on quantitative filter paper. The treatment with Lloyd's reagent and filtration was repeated. The product was practically free from color and proved to be without vitamin A activity, since it failed to restore growth and cure ophthalmia when fed to six depleted rats at a level of 30% in a vitamin A-free diet.

For the tests, the carotene or vitamin A was dissolved in the fats so that 1 gm. of ration contained 12 micrograms of carotene or 12 blue units of vitamin A as halibut liver oil.[6] Most of these rations were kept at 0°C. in the dark, in completely filled glass containers, and were prepared fresh every 4 days. However, in view of the instability of carotene in decolorized butterfat, rations containing such butterfat were kept at a temperature of —8°C.

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[4] See footnote 3, page 92.

[5] Covo brand, made by hydrogenating cottonseed oil to an iodine number of approximately 70.0. The saponification number was 196.0.

[6] The halibut liver oil was a commercial brand known as Haliver Oil furnished by the Abbott Laboratories.

Six rats were placed on each of the diets. They were kept in separate cages and were fed equivalent amounts of food; 5 gm. daily the first week; 6 the second, 7 the third, and 8 gm. daily thereafter. This amount of food was consumed shortly after being given, thus minimizing the possibility of destruction in the food cup. At the end of 5 weeks the livers were analyzed for vitamin A by the $SbCl_3$ technic (Carr and Price, '26) described previously (Lease, Lease, Weber and Steenbock, '38), and the results expressed as blue units (Moore, '30).

Marked increases in the liver stores of vitamin A resulted from the administration of either carotene or vitamin A, regardless of the kind of fat in the basal diet (table 1). Initial stores averaged 50 to 75 blue units of vitamin A per liver; final stores, 540 blue units (502 to 640). The character of the fat failed to influence the storage of vitamin A at these relatively high levels of intake.

TABLE 1

Liver stores of vitamin A produced by carotene fed in various fats

TYPE OF FRESH FAT	AVERAGE BLUE UNITS OF VITAMIN A PER LIVER [1]	
	Fed 12 micrograms of carotene per gram of ration	Fed 12 blue units of vitamin A per gram of ration
Lard (Swift's Premium)	566	508
Covo	548	579
Soy bean oil	542	640
Wesson oil	502	530
Butterfat (free of vitamin A and coloring matter)	574	537

[1] Each figure is an average of results from six animals.

Growth response to carotene. Considerable carotene was excreted when sufficient amounts were fed to produce hepatic stores of vitamin A. Hence a modifying effect of the fat may have been masked by the presence of excess carotene. The possible influence of various fats on carotene utilization was, therefore, studied at low levels of carotene intake, using the growth response and the cure of ophthalmia of depleted rats as criteria.

Carotene dissolved in decolorized butterfat was stable for 2 months when stored in vacuo at —8°C., but was markedly unstable in decolorized butterfat. When stored in small corked flasks from which the air had been partly displaced by bubbling nitrogen through the solution, 80% of the carotene was destroyed within a week at 0°C. In contrast with this, carotene was completely stable in most other oils under similar storage conditions. The degree of instability in decolorized butterfat varied with the severity of the decolorizing treatment. Thus, carotene was relatively stable in butterfat treated with amounts of Lloyd's reagent just sufficient to remove the color in 20 minutes at 45°C. Such a preparation, however, was not suitable as a medium for assaying carotene biologically because it still contained enough vitamin A to cure ophthalmia and promote growth when fed at a 20% level.

For the growth experiments, rats 35 to 50 gm. in weight were fed our vitamin A-low basal ration (heated casein 18, modified Osborne and Mendel salts 4, hydrogenated vegetable fat 10, brewers' yeast 7.9, irradiated yeast 0.1, and sucrose 60) until symptoms of ophthalmia appeared and growth ceased. They were then transferred to a similar low-A ration containing only 3% of fat, the fat being the same as that in which the supplement was administered, namely, cottonseed oil or decolorized butterfat. Carotene was fed daily as a separate supplement dissolved in the oils so that 1 to 2 drops contained 1 microgram of carotene. One or 2 micrograms of carotene were fed daily. The supplements were fed for 8 weeks.

Since the object of the study was a search for factors other than stability which might influence the biological efficiency of carotene, a rigid check was kept on the carotene content of the oils used. This was particularly necessary for the decolorized butterfat solutions because their carotene content was steadily decreasing. Fresh solutions were made up every 5 to 6 days and the carotene content determined spectrophotometrically (Baumann and Steenbock, '33) every third day. The number of drops fed per week was adjusted according to the results of the analyses, so that 7 or 14 micrograms of carotene were consumed per rat per week.

The more stable solutions, such as carotene in cottonseed oil, were prepared every second week, and stored under nitrogen at —8°C. in the dark. Aliquots were removed to a smaller flask twice a week. The carotene content of the solutions was determined spectrophotometrically at weekly intervals.

In spite of the marked difference in the stability of carotene in cottonseed oil as compared with decolorized butterfat, an identical growth response resulted from the administration of equal amounts of carotene in the two fats (table 2). This identity was observed at both the 1 and 2 microgram levels of intake. There was evidently no need to postulate a difference in the biological efficiency of carotene in these two media, when adequate precautions were taken to ensure an equality of intake.

The growth response of depleted rats to daily supplements of 1 microgram of carotene in various other oils was determined for cottonseed oil,[7] lard, coconut oil, mineral oil, soy bean oil, linseed oil, triolein, and two samples of peanut oil. One of the peanut oils was a 'refined' oil obtained commercially and the other a crude oil prepared in the laboratory by cold-pressing raw peanuts. In addition, 2 micrograms of carotene in refined peanut oil were fed daily, and 5 micrograms daily in cottonseed oil and in mineral oil. The triolein was prepared by heating molecular proportions of glycerol and oleic acid by the method of Garner ('28). The oils were fresh and of good quality. The peroxide numbers ranged from 0.08 for the decolorized butterfat to 0.94 for the lard. The free fatty acid content varied from 0.04% (expressed as oleic acid) for the cottonseed oil to 0.94% for the decolorized butterfat.

Bertram ('37) reported the presence of small amounts of carotene in several oils including linseed and soy bean oil. Our samples of these oils were also found to contain some carotene. Concentrates prepared from these oils revealed vitamin A activity. These findings may explain the reported

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[7] See footnote 3, page 92.

TABLE 2

The relative vitamin A potency of carotene fed in Wesson oil and decolorized butterfat

SUPPLEMENT	AVERAGE GAIN IN WEIGHT AT END OF						
	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
	gm.	*gm*	*gm*	*gm.*	*gm.*	*gm.*	*gm.*
1 microgram carotene per day, decolorized butterfat (4 rats)[1]	12.8 (8–19.5)	21.8 (17–34)	35.5 (25–47)	45.7 (31–65)	52.2 (43–63)	58.1 (47–69)	66.1 (56–75)
1 microgram carotene per day, Wesson oil (6 rats)	2.5 (—9–12)	21.0 (11-32)	33.4 (27–42)	45.7 (35–58)	58.0 (51–71)	62.7 (53–82)	68.7 (62–92)
2 micrograms carotene per day, decolorized butterfat (6 rats)	8.3 (3– —13)	25.0 (21–30)	39.2 (31–50)	47.6 (32–62)	58.7 (44–81)	68.1 (42–103)	78.0 (51–119)
2 micrograms carotene per day, Wesson oil (6 rats)	13.0 (10–18)	27.0 (21–32)	45.6 (34–58)	56.0 (44–68)	63.3 (51–84)	73.7 (57–92)	80.0 (62–99)

[1] Two animals from this group were discarded because of respiratory trouble.

biological superiority of linseed oil as a solvent for carotene (Basu, '37). We accordingly did not use these oils in the basal ration, and applied a correction factor for pigments when they were used as solvents for feeding carotene in small amounts.

Considerable differences in growth were obtained when equal amounts of carotene were fed in the various oils (table 3). Essentially the same amount of growth, which was quite substantial, was obtained with coconut oil, cottonseed oil, lard, soy bean oil and cold-pressed peanut oil, 55 to 79 gm. in 5 weeks, but growth on the other oils was definitely inferior.

TABLE 3

The growth response of rats to carotene fed in various media

MEDIA	NUMBER OF RATS	CAROTENE GIVEN DAILY	AVERAGE GAIN AT END OF					RANGE AT END OF 5 WEEKS
			1 week	2 weeks	3 weeks	4 weeks	5 weeks	
		micrograms	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Cottonseed oil	14	1	4	22	37	44	63	45 to 83
Coconut oil	10	1	3	19	35	47	55	40 to 83
Lard	10	1	10	25	41	53	63	40 to 94
Peanut oil (cold pressed)	6	1	11	32	50	67	79	59 to 105
'Refined' peanut oil (commercial)	11	1	7	13	22	32	36	3 to 70
Soy bean oil	3	1	11	31	46	61	70	50 to 82
Linseed oil	4	1	5	20	29	35	40	29 to 56
Triolein	10	1	7	19	27	35	40	29 to 47
Refined peanut oil (commercial)	4	2	7	27	48	58	60	49 to 70
Cottonseed oil	4	5	19	39	57	70	79	57 to 101
Mineral oil	4	5	−8	−11	−15	−17	−37	−18 to −55

The inferiority of the 'refined' peanut oil was observed in two experiments carried out at two different times. It was even more pronounced than the growth averages indicated, since these values represented only the averages for those animals which survived the 5-week period. Four other animals had died without recovering from the symptoms of ophthalmia. Many developed severe respiratory infections, notwithstanding the fact that the animals were actually in better physical condition than those on the other oils when the supplement was first administered. Only an occasional animal from the other groups died during the experimental period, except in the mineral oil group which never recovered from the effects of the vitamin A deficiency, even though 5 micrograms of carotene were fed daily. This latter is in line with the well-known experience of others and merits no further discussion.

The poor growth observed with the supplements of carotene in 'refined' peanut oil, triolein and linseed oil may have been due to a number of factors. There was no evidence that it was due to poor absorption. When rats were given 20 mg. of carotene daily in 'refined' peanut oil in comparison with cottonseed oil, no difference in the carotene excretion was observed. Furthermore when the stability of carotene in the different fats was determined at temperatures of 0, 20 and 37°C. greater stability was observed with peanut oil than with coconut oil (table 4) although growth was better on the latter. As a matter of fact too much emphasis should not be given to the weight increments as an index of carotene or vitamin A values because they may well have been determined by organoleptic or other properties of the oil inimical to physiological well-being, with the further possibility that their harmful effect was accentuated by the low level of vitamin A metabolism. Use was accordingly made of the degree and the rate of cure of ophthalmia as a more specific index. Practically all of the rats had incipient ophthalmias when the carotene solutions were given but occasionally responsiveness to carotene was delayed by respiratory infections. Data on animals thus infected were generally discarded.

The data on the cure of ophthalmia indicated that the relatively poor growth on 'refined' peanut oil, linseed oil and triolein was not due to a deficiency of vitamin A. Cures on these fats were obtained on the average in 15 to 27 days. On lard, soy bean oil, Wesson oil and coconut oil, as examples of the oils more efficient for growth, cures were obtained in

TABLE 4

Stability of carotene in various fats

SOLVENT	PER. NO.	FREE FATTY ACIDS	INITIAL CONCENTRATION	PER CENT CAROTENE REMAINING											
				1 week			2 weeks			1 month			2 months		
				0°	20°	37°	0°	20°	37°	0°	20°	37°	0°	20°	37°
		%	*micro-grams/cc.*												
Butterfat	0.05	0.19	108		90	100	94	88	88	94	94	89	96	89	66
Treated [1] butterfat	0.08	0.94	98.5	14	13	8	5	8	3	Tr	Tr	Tr	0	0	0
Cottonseed oil	0.15	0.04	84	96	100	100	97	100	84	97	92	83	97	83	68
Treated [1] cottonseed oil	0.12	0.04	109	94	94	85	87	92	76	95	95	77	92	83	66
Fresh lard	0.94	0.12	91	76	81	88	83	69	69	87	73	42	82	52	25
Coconut oil	0.54	0.05	92.5	84	91	69	84	88	11	93	56	0	69	10	0
'Refined' peanut oil (commercial)	0.65	0.12	93.5	84	81	68	90	72	45	89	72	45	76	67	19

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[1] Treated with Lloyd's reagent.

about the same time, viz., 12 to 23 days. Furthermore at least one failure to effect a complete cure of ophthalmia in 30 days resulted with each of the fats with the exception of the cold-pressed peanut oil with which complete cures were obtained in 12 days. As rats on this fat were started promptly on the carotene supplement in anticipation of a poor response, their average was not included with the others. We do not feel justified in crediting this crude peanut oil with superior attributes except insofar as it was superior to the 'refined' oil in the promotion of growth.

It was also interesting to note that treatment of cottonseed oil with Lloyd's reagent had no effect on the stability of carotene although such treatment induced properties in butterfat which were later destructive.

SUMMARY

We did not succeed in confirming the work of others that decolorized butterfat gives growth promoting properties to carotene inferior to those obtained with cottonseed oil. When excessive amounts of carotene or vitamin A were fed in lard, soy bean oil, cottonseed oil, devitaminized butterfat and a hydrogenated vegetable fat,[8] no marked differences in storage were obtained. Approximately equal growth responses were obtained when 1 microgram of carotene daily was fed in cottonseed oil, soy bean oil, lard, decolorized butterfat, coconut oil or crude peanut oil. Inferior growth was obtained on triolein, linseed oil and 'refined' peanut oil, but the rate of cure of ophthalmia was essentially the same on all oils.

LITERATURE CITED

BASU, N. K. 1937 Vitamin A and fat metabolism. Zeit. f. Vitaminforsch., vol. 6, p. 106.

BAUMANN, C. A., AND H. STEENBOCK 1933 Fat-soluble vitamins. XXXVII. The stability of carotene solutions. J. Biol. Chem., vol. 101, p. 561.

BERTRAM, S. H. 1937 Carotene in fats and oils. Öle, Fette, Wachse, Seife, Kosmetik. No. 8, p. 1; from Chem. Abst., vol. 31, p. 7680.

CARR, F. H., AND E. A. PRICE 1926 Colour reactions attributed to vitamin A. Biochem. J., vol. 20, p 497.

---

[8] A mixture of partially hydrogenated vegetable fats marketed under the trade name 'Spry.'

Coward, K. H. 1936 The utilization by the rat of vitamin A and carotene administered in different media. Biochem. J., vol. 30, p. 1878.

Dann, W. J. 1934 The transmission of vitamin A from parents to young in mammals. III. Effect of the fat content of diet during pregnancy on the transmission of vitamin A to the foetal rat. Biochem. J., vol. 28, p. 634.

De, N. K. 1936–1937 The assimilation of vitamin A and carotene by rats from some common foods with a note on the conversion factor I.U./E, proposed by the International Vitamin Conference. Ind. J. Med. Res., vol. 24, p. 751.

Drummond, J. C., B. Ahmad and R. A. Morton 1930 Further observations on the relation of carotene to vitamin A. J. Soc. Chem. Ind., vol. 49, p. 291T.

Dulière, W., R. A. Morton and J. C. Drummond 1929 The alleged relation of carotin to vitamin A. J. Soc. Chem. Ind., vol. 48, p. 316T.

Dutcher, R. A., P. L. Harris, E. R. Hartzler and N. B. Guerrant 1934 Vitamin studies. XIX. The assimilation of carotene and vitamin A in the presence of mineral oil. J. Nutrition, vol. 8, p. 269.

Dyer, F. J., K. M. Key and K. H. Coward 1934 The influence of the solvent on the vitamin A activity of (a) carotene and (b) cod-liver oil. Biochem. J., vol. 28, p. 875.

Garner, T. L. 1928 Formation of glycerides and their isomerides. J. Soc. Chem. Ind., vol. 47, p. 278T.

Great Britain Medical Research Council 1935 Reports on Biological Standards. IV. The standardization and estimation of vitamin A. Special Report series 202. (Edited by E. M. Hume and H. Chick.)

Hawk, P. B., and B. L. Oser 1931 A modification of the Osborne-Mendel salt mixture. Science, vol. 74, p. 369.

Hume, E. M., and I. Smedley-MacLean 1930 Some further notes on the relation of carotene to vitamin A. Lancet, vol. 1, p. 290.

Jackson, R. W. 1931 The effect of mineral oil administration upon the nutritional economy of fat-soluble vitamins. I. Studies with the vitamin A of butter fat. J. Nutrition, vol. 4, p. 171.

———— 1934 The effect of mineral oil administration upon the nutritional economy of fat-soluble vitamins. II. Studies with the vitamin A factor of yellow corn. J. Nutrition, vol. 7, p. 607.

Kraybill, H. R., and C. L. Shrewsbury 1936 The relative vitamin A potency of carotene fed in butterfat and cottonseed oil. J. Nutrition, vol. 11, p. 103.

Lathbury, K. C., and G. N. Greenwood 1934 The influence of the solvent on the biological effect of carotene and vitamin A. Biochem. J., vol. 28, p. 1665.

Lease, E. J., J. G. Lease, J. Weber and H. Steenbock 1938 Destruction of vitamin A by rancid fats. J. Nutrition, vol. 16, p. 571.

Moore, T. 1930 Vitamin A and carotene. V. The absence of the liver oil vitamin A from carotene. VI. The conversion of carotene to vitamin A in vivo. Biochem. J., vol. 24, p. 692.

Olcovich, H. S., and H. A. Mattill 1931 The unsaponifiable lipids of lettuce. I. Carotene. J. Biol. Chem., vol. 91, p. 105.

A QUANTITATIVE STUDY, BY MEANS OF SPECTROGRAPHIC ANALYSIS, OF ZINC IN NUTRITION [1]

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ONE FIGURE

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Whether zinc is an essential element in human nutrition is a debated question. Bertrand and Bhattacherjee ('34) reported that mice on a synthetic diet containing less than 0.05 mg. per 100 gm. died in 14 to 23 days, while littermates receiving 2 mg. per 100 gm. lived 57 to 74 days. Newell and McCollum ('33) observed no difference in growth between the rats receiving zinc and those on a zinc-free diet. This lack of difference is attributed by Stirn, Elvehjem and Hart ('35) to incomplete removal of the zinc from the casein used in the ration. Newell and McCollum ('33) state that only the crudest quantitative estimates were attempted by the spectrographic method. They accepted the absence or presence of the 4811 Angstroms line, the one which was present when 0.001 mg. of zinc was present in a 10-gm. sample, as indicating zinc-free diets since it was less than 1 part per million. Using the same spectrographic technic employed by Newell and McCollum ('33), Bayle and Amy ('27) were able to detect even smaller amounts of zinc. This suggests that the zinc content of the test ration used by Newell and McCollum ('33) introduced a supply adequate to meet the requirements of the

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[1] The data of this study are taken from a dissertation submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in nutrition, Child Welfare Research Station, State University of Iowa.

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growing animal. Furthermore, Stirn, Elvehjem and Hart ('35) were unable to remove the zinc from milk by the method of electrodialysis, the method employed by Newell and McCollum. They were able, however, to get a zinc-low milk serum product by precipitation with ammonium hydroxide. And, since it was difficult to remove the zinc from the casein, these authors used egg-white with a small amount of casein to provide the protein of the diet. When the resulting zinc-low diet was fed, growth inferior to that obtained with the added zinc was observed.

Hubbell and Mendel ('27) obtained better growth with the addition of 0.02 mg. of zinc to a low zinc diet containing 0.00005 mg. per day than with the zinc-low diet itself. The growth did not, however, equal the growth of the control animals whose diet was an ordinary laboratory ration which was estimated to contain 0.318 mg. of zinc daily. Since the addition of even greater amounts of zinc, 0.04 mg., give less marked growth than the 0.02 mg., the authors concluded that it is possible that the value of zinc lies not alone in the metal itself, but that it may in someway be associated in function with other metals present in small amounts.

The findings of Bertrand and Bhattacherjee ('34) and the later work of Todd, Elvehjem and Hart ('34) confirm the conclusion that zinc is an essential dietary constituent.

That many of the common foods consumed by man are rich in zinc is evident from the published analyses of Fairhall ('26) and Bertrand and Benzon ('28). The former estimated that man ingested about 10 to 15 mg. of zinc per day. Weitzel ('14, '19), Rost and Weitzel ('19) and Rost ('21) found that zinc in human urine ranged from 0.6 to 1.6 mg. per day, with a total elimination from urine and feces of 3.0 to 19.0 mg. Drinker, Fehnel and March ('27) reported the zinc content of the urine of adults as containing from 0.25 to 2.0 mg. per day. The fecal content of these same individuals ranged from 2.67 to 19.9 mg. per day. When foods were selected to increase the zinc content of the diet, urinary excretion did not increase appreciably while the fecal elimination corresponded more nearly with the intake, frequently extending over a period of several days.

The association of zinc with the metabolism of carbohydrates, which has been reported since this study was undertaken, intensifies the need for zinc metabolism studies. At the time the present study was undertaken it was thought that if zinc is necessary for physiological functioning, it might be determined by means of balance studies with children since during growth zinc should be retained in appreciable amounts. The purpose of this study was to determine how much, if any, zinc is retained by children of preschool age. The zinc lines of the spectrograms of ashed food, feces, urine, distilled water and acid alcohol were compared with spectra produced by solutions of known zinc content.

MATERIALS AND METHODS

The diets employed, the care used in handling and collecting of food and excreta, the preparation of aliquots for spectrographic analysis and the method used in reading the spectrograms is given by Scoular ('38).

Since the wedge spectrograms used in the copper study were also used to obtain the zinc retentions, all precautions which were employed to prevent contamination with copper, also prevented the addition of extraneous zinc.

For the quantitative determination of zinc two standard solutions were prepared to serve as checks. One was made by dissolving a known weight of pure zinc ribbon[3] in hydrochloric acid, and the other by dissolving a known weight of zinc precipitated for the purpose of purification by 8-hydroxyquinoline[4] in hydrochloric acid. Both were made up to volume with redistilled water, so that 1 ml. contained 1.01 mg. of zinc. When the spectrograms were made of the two solutions, the lengths of the zinc lines, 3345 Angstroms, agreed within the reading error of 0.2 mm.

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[3] Pure zinc ribbon furnished Waldbauer and Gantz ('33) by the Jersey Zinc Company.

[4] Method described in Organic Reagents for Metals, Staff of Research Laboratory of Hopkins and Williams, Ltd., 1933.

The effect of the sodium chloride, present in the ash of the unknowns, upon the length of the copper line suggested that it might also have some effect upon the zinc lines. Consequently, two sets of electrodes were prepared from the spectrographic quality graphite, both of which contained the same concentration of zinc, but one had in addition to the zinc, 4 drops of a saturated solution of pure sodium chloride. When the spectrograms of these two sets of electrodes were compared, it was found that there was less difference between the zinc line 3345 Angstroms and the reference bands of 3889 Angstroms when the electrode contained sodium chloride. This effect was similar to that observed with copper when sodium chloride was present in the electrode. Therefore, both zinc lines, with and without the addition of sodium chloride, were plotted for a given concentration (fig. 1).

The selection of the zinc line to be read in obtaining the differences between its length and that of the reference bands, 3889 Angstroms, offered no problem with the standard solutions. But in the case of the unknown solutions it was found that the sodium line, 3303 Angstroms, which was present in the spectra of all food, feces and urine samples, masked the zinc lines 3302.9 and 3302.6 Angstroms. The zinc line 2139 Angstroms in the ultraviolet region could not be read because plates sensitized for that region had not been used. Since the triple band at 4680–4722–4810 Angstroms was not clear, reported by Lamb ('35) to be due to the incandescent effect of the graphite after the residue had been burned out, the lines 3345.0–3345.5 Angstroms, which were never separated but considered as 3345 Angstroms in this study, were used in reading the lines of all spectrograms. Some difficulty was experienced in getting clear-cut measurements due to the calcium line 3344.5 Angstroms which was present in some spectra, its concentration being such that it tended to blur the ends of the zinc lines, thus making it difficult to get reading checks within 0.2 mm. To lessen the error when this was encountered, three or more readings were made and the average difference used in comparing concentrations of duplicate samples.

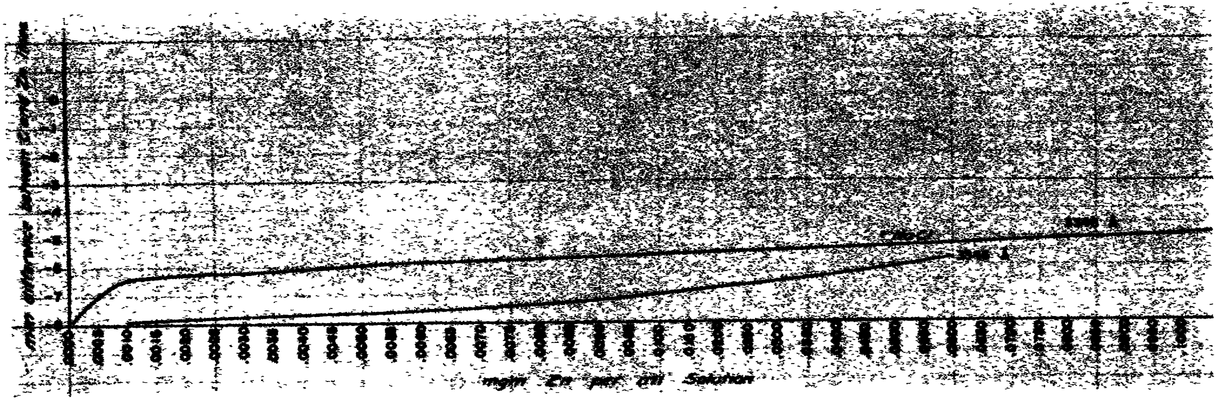

Fig. 1 Difference between the length of the zinc line 3345 Angstroms and the reference bands 3889 Angstroms plotted for each concentration of zinc, with and without added sodium chloride.

The 'best-fit' line, as described by Scoular ('38) in the copper study, was used in determining the concentration of zinc in the unknown solutions. In this way the zinc concentration was read from the line (fig. 1) which most nearly approximated the sodium chloride present in the unknowns. The feces offered the most difficulty in reading, due to the tendency of the quantity of calcium present to overshadow the zinc lines. These technical difficulties are well illustrated by the zinc recovery values from fecal samples which are given in table 1. The addition of 0.005 mg. of zinc to the electrodes containing the fecal ash gave recoveries of from 200 to 250% before these solutions were diluted, and from 90 to 100% after dilution. The high calcium concentration prior to dilution, was believed to be the primary cause of these high figures since the other recoveries had been made with samples containing much less zinc. The difference in the analyzed concentration of zinc in the fecal samples before and after dilution, which varied from 0.000 to +0.001 mg., indicated the

TABLE 1

Amount of added zinc recovered from samples of food, feces and urine

PERIODS	SAMPLES TESTED, CONCENTRATION	SAMPLE PLUS 0.005 MG Zn		
		Concentration	Recovery	
	mg.	*mg.*	*mg.*	%
Urine				
G.E. 2/9	0.0010	0.0060	0.0050	100
F.V. 2/9	0.0005	0.0060	0.0055	110
J.E. 2/9	0.0009	0.0060	0.0051	102
Feces				
G.E. 2/9	0.0400	0.0500	0.0100	200
F.V. 2/9	0.0500	0.0625	0.0125	250
J.E. 2/9	0.0600	0.0725	0.0125	250
	Same samples of feces diluted 1:10 with redistilled water			
G.E. 2/9	0.0040	0.0085	0.0045	90
F.V. 2/9	0.0060	0.0110	0.0050	100
J.E. 2/9	0.0060	0.0110	0.0050	100
Food				
G.E. 2/9	0.0068	0.0125	0.0057	114
F.V. 2/9	0.0055	0.0100	0.0045	90
J.E. 2/9	0.0055	0.0110	0.0055	110

correctness of this assumption. The average range of error for Fairhall and Richardson's ('30) turbidimetric method, which was used by Newell and McCollum ('33) when quantitative determinations were desired, was given as 0.060 mg. Therefore, it was assumed that the concentration of zinc obtained from the readings of the spectra which were made from undiluted solutions of fecal ash was valid and dilution was resorted to only when recoveries were desired.

RESULTS AND DISCUSSION

The spectrograms of distilled water used for drinking purposes showed that the water contained a uniform amount of zinc, namely, 0.001 mg. per 500 ml. The acid-alcohol used on the food and feces, however, contributed from 0.002 to 0.010 mg. per 100 ml., the alcohol of the solution being the source of the zinc. Newell and McCollum ('33) similarly found that alcohol was a source of zinc contamination in one batch of casein in which alcohol was used in purification.

Spectrographic analysis of the diets given in table 2 shows that the ingestion of zinc by the children studied varied from 4 to 6 mg. This variation apparently was not related directly to the changes introduced in the several diets but rather to the varied amounts contained in staple foods. The theoretical intake which was calculated on the basis of the analyses of Fairhall ('26) and Bertrand and Benzon ('28), suggested that diet I (Scoular, '38) contained less zinc than diet II, and that the latter was not as rich as diet III. All of the estimated values, however, were from 3.5 to 3.6 mg. higher than those obtained by spectrographic analysis. The average difference between the zinc content of the three diets as estimated and as analyzed, were almost identical, being 1.3 mg. for the spectrographic method and 1.4 for the theoretical estimate. The close relationship to these values suggest that the differences are due to methods. In the present study the highest ingestion level was only 41% higher than the lowest ingestion. From an inspection of table 2, the differences in zinc composition of the individual diets, within this limited range of

Average daily intake, excretion and retention of zinc

DIET NO.	CASE DATE	ZINC INGESTION					ZINC EXCRETION				RETENTION	
		Food		A.A.	Total		Feces	Urine	A.A.	Total	Total	Per kg.
		Theoretical[1]	Analyzed		Mg.	Per kg.						
		mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg*	*mg.*	*mg.*	*mg.*
II	F.V. 3/22	7.79	4.48	0.01	4.47	0.218	3.44	0.01	0.01	3.46	1.01	0.045
I	J.E. 2/9	7.83	4.29	0.01 [2]	4.28	0.222	4.66	0.02	0.00 [2]	4.68	—0.40	—0.019
I	F.V. 2/9	7.57	4.22	0.01 [2]	4.21	0.222	4.33	0.01	0.00 [2]	4.34	—0.13	—0.005
II	G.E. 3/22	7.46	3.81	0.01	3.80	0.223	6.40	0.10	0.00	6.50	—2.70	—0.159
III	F.V. 6/7	8.60	4.91	0.01	4.90	0.223	3.43	0.16	0.00	3.59	1.31	0.063
III	F.V. 5/12	8.60	4.58	0.01	4.57	0.224	4.46	0.21	0.00	4.67	—0.10	—0.005
I	J.E. 2/4	7.83	4.37	0.01 [2]	4.36	0.226	4.07	0.14	0.00 [2]	4.21	0.15	0.008
I	F.V. 2/4	7.57	4.32	0.01 [2]	4.31	0.227	4.27	0.03	0.00 [2]	4.30	0.01	0.001
III	F.E. 5/17	8.60	4.68	0.01	4.67	0.227	2.27	0.28	0.00	2.55	2.12	0.112
II	G.E. 3/18	7.46	3.89	0.01	3.88	0.229	2.41	0.01	0.00	2.42	1.46	0.082
II	J.E. 3/22	7.99	4.56	0.01	4.55	0.230	3.09	0.17	0.00	3.26	1.29	0.069
III	J.E. 5/12	8.67	4.79	0.01	4.78	0.234	4.30	0.09	0.00	4.39	0.39	0.019
III	J.E. 4/21	8.64	4.79	0.01	4.78	0.237	2.02	0.02	0.00	2.04	2.74	0.135
II	G.E. 3/1	7.46	4.04	0.02	4.02	0.239	6.62	0.01	0.01	6.64	—2.62	—0.131
III	F.V. 6/2	8.60	5.01	0.01	5.00	0.240	3.74	0.07	0.00	3.81	1.19	0.056
III	J.E. 5/17	8.67	4.95	0.01	4.94	0.241	5.44	0.13	0.00	5.57	—0.63	—0.030
III	J.E. 4/26	8.64	4.88	0.01	4.87	0.241	4.72	0.04	0.00	4.76	0.11	0.011
III	J.E. 6/7	8.67	5.09	0.01	5.08	0.246	4.37	0.00	0.00	4.37	0.71	2.033
III	F.V. 4/26	8.58	5.51	0.01	5.00	0.246	2.90	0.01	0.00	2.91	2.09	0.101
II	F.V. 3/1	7.79	4.85	0.02	4.83	0.249	6.52	0.01	0.01	6.54	—1.71	—0.091
III	G.E. 4/26	7.48	4.29	0.01	4.28	0.250	5.08	0.09	0.00	5.17	—0.89	—0.023
II	J.E. 3/17	7.99	4.94	0.01	4.93	0.250	4.61	0.16	0.00	4.77	0.16	0.010
III	J.E. 6/2	8.67	5.19	0.01	5.18	0.251	3.70	0.16	0.00	3.86	1.32	0.064
III	F.V. 4/21 [3]	8.58	5.15	0.01	5.14	0.254	3.44	0.01	0.00	3.45	1.69	0.084
III	G.E. 6/7	7.50	4.61	0.01	4.60	0.258	4.93	0.25	0.00	5.18	—0.58	—0.033
II	F.V. 3/19	7.79	5.05	0.01	5.04	0.259	3.99	0.01	0.00	4.01	1.03	0.050
III	G.E. 5/12	7.50	4.58	0.01	4.57	0.262	4.29	0.12	0.00	4.41	0.16	0.004
III	G.E. 4/21	7.48	4.56	0.01	4.55	0.264	4.37	0.01	0.00	4.38	0.17	0.010
II	J.E. 3/1	7.93	5.08	0.02	5.06	0.266	6.27	0.01	0.01	6.29	—1.23	—0.063
III	G.E. 5/17	7.50	4.74	0.01	4.73	0.269	4.50	0.18	0.00	4.68	0.05	0.004
I	G.E. 2/9	7.31	4.65	0.01 [2]	4.64	0.279	3.38	0.00	0.00 [2]	3.38	1.26	0.075
III	G.E. 6/2	7.50	5.03	0.01	5.02	0.282	3.38	0.01	0.00	3.39	1.63	0.094
I	G.E. 2/4	7.31	4.79	0.01 [2]	4.78	0.288	3.10	0.02	0.00 [2]	3.12	1.66	0.100
II	F.V. 2/25	7.79	5.88	0.01	5.87	0.300	2.47	0.00	0.01	2.48	3.39	0.171
II	G.E. 2/25	7.46	5.21	0.01	5.20	0.307	3.11	0.01	0.01	3.13	2.07	0.123

[1] Estimated from values published by Fairhall ('26) and Bertrand and Benzon ('28).

[2] Estimated values.

[3] Slight regurgitation which was not analyzed.

zinc ingestion, appear more significant than the actual differences warrant. For example, when the difference in the ingestion level of F.V. and J.E., two children of about the same age and weight, for period 3/22 is calculated, J.E.'s is higher by only 5%. However, when tabulated in the order of increasing zinc ingestion levels, nine cases fill the intervening gap of 5%. Since the range of error estimated for the spectrographic method used in this study approximated 10%, this difference in the zinc content of the diet for period 3/22 loses its significance. The determined zinc values of the foods used in two consecutive 5-day metabolism periods were very close, all falling within the 10% range of error.

The amount of zinc excreted in the urine was small, representing from 0.04 to 6.0% of the intake. This was not related to intake nor was it constant for a given individual. In some cases there was little change from period to period, in other instances there were wide variations.

Zinc is obviously excreted through the alimentary tract. The fecal output ranged from 2.016 to 6.624 mg. per day, representing from 42 to 163% of the ingested zinc. At times there was almost no difference between the amounts of zinc excreted in the feces of the two 5-day metabolism periods as in 2/4 and 2/9, and 3/19 and 3/22 for F.V. On the other hand, periods 5/12 and 5/17, and 2/25 and 3/1 for the same child (F.V.) varied more than 50%. These excretions bore no relation to the differences noted in the urinary excretion, nor to the amount of zinc ingested, although there was a tendency toward higher fecal zinc with the higher ingestion levels. These findings corroborate those of Drinker, Fehnel and March ('27) that urinary excretions of zinc did not increase appreciably with high zinc intake, while the fecal elimination corresponded more nearly with the intake, frequently extending over a period of several days. Some of the negative balances in the present study may have resulted from delayed excretion.

Of the thirty-five balances tabulated (table 2), ten are negative with a range of —0.005 to —0.131 mg. per kilogram of body weight. And six of these ten occurred with an ingestion level which was lower than the average (0.248 mg. per kilogram of weight) for the entire study. Of the twenty-five positive balances, the highest retention (0.171 mg.) occurred when F.V. received his highest ingestion level, namely, 0.300 mg. per kilogram of body weight. However, J.E. showed his highest retention of 0.135 mg., with an ingestion level below that of the average, namely, 0.237 mg. per kilogram of body weight. Since the two highest retentions, not including the exception just mentioned, were obtained with the two highest ingestion levels, 0.300 and 0.307 mg. per kilogram, it would seem that higher ingestion levels might give better retentions. On this basis it might be tentatively concluded that an ingestion of not less than 0.300 to 0.307 mg. of zinc per kilogram of body weight is necessary for the preschool age child.

SUMMARY

Thirty-five zinc balance studies have been made with three normal boys of preschool age.

The ashed food, feces, urine, distilled water and acid-alcohol were analyzed spectrographically for zinc by comparing their spectra with those produced by solutions of known concentration.

The zinc values for food which were analyzed by quantitative spectrographic technic were found to be almost 40% lower than the theoretical values estimated from published analyses for which the turbidimetric method had been used.

From 0.04 to 6.0% of the ingested zinc was eliminated through the urine. The rest of the excreted zinc, representing from 42 to 164% of the ingested amount, was eliminated through the alimentary tract.

Since more than two-thirds of the thirty-five balance studies gave retentions which were significantly greater than the error determined for the method used, it would seem that zinc is associated with physiological functioning.

On the basis of the limited ingestion range studied it is tentatively concluded that 0.307 mg. of zinc per kilogram of body weight will supply the zinc needs of the preschool age child.

The author wishes to express her grateful appreciation for the helpful suggestions and criticisms and for the encouragement offered by Dr. Amy L. Daniels and Dr. Louis J. Waldbauer during this study.

LITERATURE CITED

BAYLE, E., AND L. AMY 1927 Application of spectrographic analysis to the estimation of metallic impurities. Compt. rend. Acad. Sci., T. 185, p. 268.

BERTRAND, G., AND B. BENZON 1928 Zinc content of some plants. Compt. rend. Acad. agr. France, T. 14, p. 1303.

BERTRAND, G., AND R. C. BHATTACHERJEE 1934 Combined action of zinc and vitamins in animal nutrition. Compt. rend., T. 198, p. 1823.

DRINKER, K. R., J. E. FEHNEL AND M. MARCH 1927 Normal excretion of zinc in the urine and feces of man. J. Biol. Chem., vol. 72, p. 375.

FAIRHALL, L. T. 1926 Estimation of zinc in biological materials. J. Ind. Hyg., vol. 8, p. 165.

FAIRHALL, L. T., AND J. R. RICHARDSON 1930 Nephelometric analysis of zinc. J. Am. Chem. Soc., vol. 52, p. 938

HARRISON, G. H. 1936 Practical possibilities in spectrographic analysis. Metals and Alloys, vol. 7, p. 291.

HUBBELL, R. B., AND L. B. MENDEL 1927 Zinc and normal nutrition. J. Biol. Chem., vol. 75, p. 567.

LAMB, F. W. 1935 Spectrographic determination of impurities in commercial cadmium. Proc. Am. Soc. Testing Materials, Part II, vol. 35, p. 71.

NEWELL, J. M., AND E. V. MCCOLLUM 1933 Studies on the role of zinc in nutrition. J. Nutrition, vol. 6, p. 289.

ROST, E. 1921 Woher stammt das Zink in menschlichen und tierischen Organismus. Med. Klin., Bd. 17, S. 123.

ROST, E., AND A. WEITZEL 1919 Zur Kenntnis des Vorkommens von Zink (und Kupfor) in den Ausscheidungen und Organen des Menschen und in unser Lebensmitteln. Arb. Reichsgsndhtsamte, Bd. 51, S. 494.

SCOULAR, FLORENCE I. 1938 A quantitative study by means of spectrographic analysis of copper in nutrition. J. Nutrition, vol. 16, p. 437.

STIRN, F. E., C. A. ELVEHJEM AND E. B. HART 1935 Indispensability of zinc in nutrition of the rat. J. Biol. Chem., vol. 109, p. 347.

TODD, E. R., C. A. ELVEHJEM AND E. B. HART 1934 Zinc in the nutrition of the rat. Am. J. Physiol., vol. 107, p. 146.

WEITZEL, A. 1914 Über das naturliche Vorkommen von Zink in Lebensmitteln und in the Ausscheidungen of Menschen. Zentr. Physiol., Bd. 28, S. 766.

——— 1919 Beitrage zur Bestimmung von Zink in organischen Stoffen—Harn, Kot, Lebensmitteln usw.—nebst Bemerkungen über den Zinkgehalt von Reagentien und Analysengefassen. Arb. Reichsgsndhtsamte, Bd. 51, S. 476.

RAT ACRODYNIA AND THE ESSENTIAL FATTY ACIDS [1]

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The first dietary studies which suggested a relationship between dermal abnormalities and fat deficiency were made by Burr and Burr ('29, '30). On their diet which contained ether-extracted yeast, rats developed mild skin lesions, consisting of scaliness of paws and tail, in 8 to 10 weeks. The symptoms were healed by the administration of unsaturated fats, or specifically, esters of linoleic acid. Hogan and Richardson ('34), and Richardson and Hogan ('36), using a lower level of yeast which had been irradiated, obtained severe symptoms in 5 to 6 weeks. The use of either cornstarch or rice starch in the diet prevented their occurrence; butterfat likewise delayed or prevented them. Cures were produced with fats, especially wheat germ oil, but since the activity of the fats did not parallel their unsaturation, the curative properties were not believed to be due to a fatty acid. Cures were also obtained with aqueous solutions and their fuller's earth adsorbates. György ('35) produced dermatitis, later called rat-acrodynia, with fair success on a diet consisting chiefly of rice starch and containing 9% butterfat. The symptoms did not appear until the seventh to the eighth week, however, and in some cases disappeared spontaneously.

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[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station, Madison, Wisconsin.

The most potent curative materials tested were Peter's eluate, equivalent to 10 gm. of baker's yeast daily, and wheat germ. While Birch and György ('36) had difficulty in getting the entire activity into aqueous solution, they were able to prepare concentrates by adsorption on fuller's earth and by electrodialysis. Cures with lard or linseed oil were also reported. Halliday and Evans ('37), using sucrose and only 3% butterfat in the diet, reported the production of a dermatitis within 3 to 4 weeks. Lepkovsky, Jukes and Krause ('36) using an almost identical diet except that it contained 10% lard did not obtain dermatitis unless they added a 'filtrate factor,' factor 2. Cures were obtained with a fuller's earth adsorbate, factor 1. Bender, Ansbacher, Flanigan and Supplee ('36), and Bender and Supplee ('37) found the production of dermatitis irregular on a diet containing 10% hydrogenated cottonseed oil,[2] but upon decreasing the fat to 3% obtained symptoms consistently. Salmon ('38) reported that oils alone or starch alone failed to cure or prevent acrodynia and that oils did not contain the entire dermatitis-preventing factor. Nor did the methyl esters of linoleic and linolenic delay the onset of symptoms. Birch ('38) more recently reported that two factors were concerned: one, water soluble, viz., vitamin B_6 and one fat soluble similar to the fatty acid factor of Burr and Burr. It is evident from the foregoing that the relation of certain constituents of the diet to the production and cure of dermatitis is not revealed clearly. The following results with rats on various diets and fat supplements already reported briefly (Quackenbush and Steenbock, '38) are therefore presented in detail.

EXPERIMENTAL

Diets and the production of dermatitis

The various constituents of the diets are given in table 1. Casein 30 was prepared from commercial casein by extracting it with N/50 acetic acid for 1 week. It was washed free from acid, dried at 50°C. and ground, then extracted twice with

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[2] Crisco.

1.5 liters of alcohol per kilo at 45 to 50°C. for two periods of 2 days each, filtered on a large Büchner funnel and again dried.

The irradiated yeast was prepared from dried brewer's yeast (Anheuser-Busch). It was ground to pass a 100-mesh sieve, and irradiated with occasional stirring in thin layers, 400 gm. per square meter, with a large cold quartz burner at 16 cm. The temperature of the yeast did not rise above 30°C. One sample was irradiated for 5 hours; another for 25 hours. One hundred milligrams of these were fed as daily supplements for diets I and II respectively.

TABLE 1

Primary constituents of diets[1]

DIETS	CASEIN 30	WESSON SALTS	CARBOHYDRATE			
	gm.	*gm*	*gm.*		*gm.*	
VI	18	4	Cerelose	68	Hydrogenated coconut	10
VII	18	4	Cerelose	68	Peanut	10
VIII	18	4	Cerelose	68	Wheat germ	10
V	18	4	Cerelose	78		
XII	18	4	Sucrose	78		
XIII	18	4	Rice starch	78		
I	20	4	Sucrose	71	Cod liver	2
II	20	4	Sucrose	71	Cod liver	2

[1] For supplements see text.

The mineral elements were supplied as Wesson salts (Wesson, '32) or salts P, composed of NaCl 5.0, $CaCO_3$ 36.0, $CaCl_2.6H_2O$ 28.4, $CaHPO_4.2H_2O$ 21.8, $FeCl_3.6H_2O$ 2.4. Agar was fed at a level of 3% in diets I and II. Daily vitamin supplements consisted of 10 micrograms B_1 (Merck's synthetic), 20 micrograms riboflavin, 10 micrograms β-carotene, and 5 micrograms calciferol, except for diets I and II. The B_1 and riboflavin were dissolved in N/50 acetic acid and fed from a medicine dropper. In all crucial experiments synthetic riboflavin (Hoffman-La Roche) was used. In routine assays, however, either a concentrate of high purity or the natural crystalline compound was used. Liquid hydrogenated coconut oil was used for the administration of calciferol and carotene and for the dilution of the more potent oils. It was prepared by crystallizing out the solid fats from a coconut oil which had been hydrogenated to an iodine number of less than 0.20. Tests showed it to be biologically inactive. One drop of the solution was given to each rat daily.

All of the animals which received either no fat or hydrogenated coconut oil developed well-defined symptoms in 8 to 10 weeks. With the addition of 10% peanut oil or wheat germ oil to the diet lesions were not produced. Since our stock diet (Steenbock, '23) consisted chiefly of natural grains, it was assumed that initial storage in the experimental animals might account for the slow rate at which dermatitis was produced. Departing from the use of grains, diet P composed of potato meal 90 gm., salts P, 1.6 gm., casein 30, 8.4 gm. was fed. The mother rat received 22 mg. halibut liver oil twice a week during the nursing period. The potatoes were steamed for 40 to 45 minutes, sliced and dried on wire screens before a fan at room temperature and then ground to a meal. Each daily portion of the diet was moistened before feeding.

TABLE 2

Incidence of dermatitis on the various diets

DIET	NUMBER OF ANIMALS	DIET DURING NURSING PERIOD	NUMBER SHOWING MILD SYMPTOMS	NUMBER SHOWING SEVERE SYMPTOMS	AVERAGE TIME FOR SEVERE SYMPTOMS TO DEVELOP *weeks*	LENGTH OF EXPERIMENT *weeks*
I	7	S	4	0		9
I	5	S	5	0		22
II	11	S	8	0		9
V	17	P		17	4½	5
VI	2	S		2	10	10
VI	5	P	1	4	4½	6
VII	7	P	0	0		7
VIII	2	P	0	0		10
XII	8	P		8	6	14
XIII	9	P	5	4	8+	14

S, stock diet. P, potato diet.

On diet V the rats developed an acute dermatitis in 4 to 5 weeks (table 2). When sucrose was used instead of cerelose the symptoms were retarded slightly. With rice starch, on the other hand, less than half of the animals developed a severe dermatitis within 14 weeks. By this time most of the animals were quite emaciated. Neither Labco nor Glaxo casein substituted for casein 30 hastened the appearance of symptoms. When irradiated yeast supplemented diets I and II similar to Hogan and Richardson ('34) diet only mild symptoms of dermatitis were produced (table 2).

The following standard technic was adopted for the production of animals for curative studies: females with litters of four to six young were transferred within 2 days after delivery, from the stock diet to diet P. Ten days later they were placed on screens. When the young reached a weight of 40 gm., usually during the fourth week, they were weaned and given diet V. After 5 weeks on this diet dermatitis developed without exception. Spontaneous recoveries were not observed.

Two well-defined symptom complexes, the acute and the chronic form, were recognized. In the acute form the lips, eyes, and fore paws were the areas chiefly involved. The animals became inactive and lost from 5 to 10 gm. in weight. Without supplement, most of the animals died within a few days.

The chronic form usually developed in animals which received supplements of low potency. The lesions were very extensive. The entire peripherae, especially the ears and hind paws became involved. The lips and fore paws were generally parchmentized. Ringlets of scales appeared on the tail which sometimes sloughed off leaving a shiny, denuded surface. The torso was usually not denuded at all, but in the chronic form it was covered with minute adherent scales. The chronic form could not be differentiated from György's 'rat-acrodynia.'

The initial nature of the dermatitis and the length of the required assay period were important factors in the determination of potencies. To evaluate them properly the symptoms were charted systematically (table 3).

Within 2 to 3 weeks after the administration of a potent supplement, the swelling and inflammation disappeared, the skin was regenerated and the hair was restored in all the areas involved. The lips and eyes healed more rapidly than the paws. The last visible symptom to disappear was a dry scaliness of the hind paws and tail, and occasionally of the fore paws. In some cases a condition of scaliness persisted for several weeks, even when the amount of supplement had been increased tenfold. This was especially noticeable with yeast supplements; and it was most persistent during the winter months, probably because of the lower humidity (Brown and Burr, '36). Accordingly, absence of scaliness was not used as a criterion of potency.

TABLE 3

Stages of dermatitis

	STAGE 1	STAGE 2	STAGE 3	STAGE 4
Lips	Hyperemia, usually with small ulcers at corners	Slight swelling, redness along vertical groove	Medium swelling, desquamation to nostrils	Severe swelling, raw soreness of wide area around mouth and nostril
Eyes	Lids wet, slight crusts at the corners	Slight swelling of lids, crusts in corners	Medium swelling, lids often adherent with gummy exudate	Severe swelling, lids swollen shut, large crusts on lids
Fore paws	Slight scaliness, redness following desquamation between digits	Slight swelling digits desquamated, red and sore	Medium swelling, digits raw and sore, sometimes with pus accumulation	Severe swelling, desquamated and sore to wrists; gangrenous
Hind paws	Same as fore paws	Same as fore paws; also desquamation of large areas on paws	Same as fore paws; skin cracked and ulcerated at heel and along tibia	Severe swelling; large red areas over paws and inner thigh, paws often gangrenous
Ears	Hyperemia, slight swelling	Pinnae swollen and thickened due to scale formation	Medium thickening at lips, large scales sloughing	Severe thickening, necrosis
Tail	Scaly (ringlets)	Very scaly	Very scaly; necrosis near tip	

The method used for quantitative assays was standardized as follows: animals were given supplements as soon as the lips and paws reached stage 2 to 3 (table 3). A cure was assumed to have been effected if at the end of a 21-day period of assay the lips, eyes and ears were normal in appearance and the paws and tail free from all symptoms except a dry scaliness. A minimum curative dose was defined as that amount which when given daily produced cures in more than 50% of the animals, with none failing to respond to twice the amount of the same supplement.

TABLE 4

Potencies of oils

OIL SAMPLE	MINIMUM CURING	MAXIMUM FAILING
	drops/day	*drops/day*
Wheat germ (commercial)	½	⅙
Wheat germ (ether extract)	⅓	...
Cottonseed (Wesson)	½	...
Corn (Mazola)	½	...
Peanut (commercial)	1	½
Peanut (ether extract)	1	...
Linseed (commercial)	2	1
Linseed (ether extract)	2	1
Lard	2	...
Olive	5	2
Butterfat	25	10
Coconut	10	5
Hydrogenated coconut	...	10
Cod liver	...	2
Halibut liver	...	⅓

The curative action of fats

With the standardized technic the minimum curative dose for wheat germ oil, peanut oil, cottonseed oil and corn oil was approximately 0.5 to 1 drop daily. Coconut oil and butterfat were far less potent, requiring 10 to 25 drops daily. Hydrogenated coconut oil was entirely inactive (table 4). It might be inferred from these results that the biological activity paralleled the degree of unsaturation. However, the results with cod liver oil and linseed oil did not support this inference;

they were both less active than the more saturated wheat germ, peanut, cottonseed and corn oils.

The permanency of the cures obtained was determined with twenty animals which had been used in routine assays. All had developed severe dermatitis in 4 to 5 weeks on diet V, and all had been cured with fat supplements by the end of the eighth week. Five of these animals were given diet V without supplement; within 4 to 5 weeks all had again developed the acute symptoms in their original severity. The other fifteen animals continued to receive small supplements of peanut oil, wheat germ oil, lard or butterfat, and at the end of the same period showed—except for an occasional scaly tail—none of the symptoms. Three of the animals which received diet V which had been supplemented with 3% peanut oil were continued on the diet for 27 weeks, without recurrence of the symptoms.

The effect of various treatments on curative properties

Oxidation. A sample of peanut oil having a peroxide number of 15 was placed in an aspiration cell placed directly below a 60-watt Mazda lamp. Air was bubbled through the oil at a temperature of 50 to 60°C. for a period of 72 hours. Although the peroxide number was increased to 34, the oil was still curative when fed at 2 drops per day.

Irradiation. A sample of peanut oil was placed under nitrogen in a quartz container and irradiated at a distance of 15 cm. with a cold quartz lamp for 10 hours. The irradiated oil produced cures when 2 drops were fed daily.

Saponification. A sample of peanut oil was saponified with KOH in alcohol under nitrogen. The reaction mixture was extracted thoroughly with ether, and the extract evaporated to dryness. The residue was diluted with hydrogenated coconut oil so that 1 drop of the mixture contained the unsaponifiable fraction from 5 gm. of peanut oil. This material failed to cure dermatitis even when 2 drops were fed daily.

The ether-extracted soaps were acidified with HCl and the fatty acids taken up with ether. After washing and drying the solution, the ether was removed under reduced pressure.

When the fatty acids were fed at a level of 3 drops per day, the animals developed severe localized irritation and failed rapidly. A portion of the fatty acids was esterified by refluxing with absolute alcohol in the presence of H_2SO_4. The ethyl esters were washed thoroughly with water and dried by warming under reduced pressure. The product was fed at 3 drops per day to animals in both the acute and chronic forms. Complete cures were obtained in all cases.

A sample of wheat germ oil was saponified and the mixed ethyl esters were prepared by the method described above. The product was found to have the same potency as the original oil. It was used for fractionations as described below. The acids from corn oil were also fractionated.

Fractional distillation of the ethyl esters from wheat germ oil

Distillates from 40 gm. of the ethyl esters prepared from wheat germ oil were collected at 1 mm. pressure at 150 to 200°C. Of the various fractions only two, namely, those distilling at 170 to 173°C. (D_2) and at 173°C. (D_3) were found to be biologically potent when given in amounts of 1 drop per rat daily. D_2 (170 to 173°C.) was the only fraction which gave some improvement at $\frac{1}{10}$ drop. These two fractions comprised 60% of the total distillate. No further concentration of potency was obtained by their redistillation.

Fractional crystallization of fatty acids from wheat germ oil

The fatty acids were crystallized from acetone according to the method of Brown and Stoner ('37). The fractions crystallizing above —25°C. were found to be biologically inert; the first potency was obtained with the fraction crystallizing at —45°; those separating between —50 and —75° showed the highest potency (table 5).

Preparation of ethyl linoleates from corn oil

Three samples of ethyl linoleate were prepared from the fatty acids of corn oil. The first was obtained from fatty acids which had been fractionally crystallized from acetone according to the method of Brown and Stoner ('37). The second was prepared from fatty acids which had been separated by bromination according to Rollett ('09). The third was obtained by the same process as the second followed by elaidinization with nitrous acid and recrystallization of the product from acetone.

Properties of the esters

The three ester preparations were colorless oils at room temperature, and all solidified upon cooling to —60°C. The ethyl α-linoleate and the ester obtained by direct fractional crystallization melted at —55°; the elaidinized product at —18°. The iodine values (Hanus) were 165, 158, 160, respectively. The data for these esters supply further evidence for the hypothesis of Brown and Frankel ('38) that linoleic acid prepared by direct fractional crystallization is identical with α-linoleic acid.

TABLE 5

Potency of the fatty acids of wheat germ oil crystallized from acetone

FRACTION	CRYSTALLIZATION TEMPERATURE	YIELD	I V. OF ETHYL ESTERS HANUS	MINIMUM CURING	MAXIMUM FAILING
	°C.	*gm.*		*drops/day*	*drops/day*
C_1	0	4.0	10	..	1
C_2	—25	25.3	70	..	1
C_3	—45	17.8	117	1	½
C_4	—50	20.2	141	½	⅒
C_5	—55	12.5	149	½	⅒
C_6	—75	8.2	153	½	⅒
C_7	Filtrate	3.2	165	1	½

When the esters were assayed for biological activity, the ethyl linoleates obtained from linoleic acid by direct crystallization and as the hexabromide were active in doses of ½ drop daily, the elaidinized ester was found to be inactive at 1 drop daily. All were inactive when given in amounts of ⅒ and ¼ drop per rat daily.

SUMMARY

On a basal diet of purified casein, glucose and salts, supplemented with carotene, calciferol, synthetic B_1 and riboflavin, rats developed skin lesions in 4 to 5 weeks; an acute dermatitis when restricted to the basal diet; and a chronic form, 'rat-acrodynia,' when given fat supplements at subcurative levels.

Complete healing of either the acute or chronic form was obtained by the administration of peanut oil or wheat germ oil. Cured animals maintained themselves free from all symptoms so long as the fat supplements were continued or until the end of the survival period, which was usually several months. Aeration or ultraviolet radiations did not destroy the activity in peanut oil. It was resident in the fatty acid fraction.

Cures were produced with ½ drop of wheat germ oil, corn oil or Wesson oil, 10 drops of coconut oil or 25 drops of butterfat. The lesions were not prevented nor cured with hydrogenated coconut oil.

The unsaponifiable fraction from wheat germ oil was devoid of potency; the ethyl esters prepared from the soap fraction contained all of the activity. When the unsaturated fatty acids were fractionally crystallized from acetone, the highest potency was obtained with the fraction separating between —50 and —75°C. One-half drop of ethyl linoleate (Rollett, '09) per day cured the dermatitis completely. The ethyl ester of elaidinized linoleic acid was found to be inactive.

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LITERATURE CITED

Bender, R. C., S. Ansbacher, G. E. Flanigan and G. E. Supplee 1936 The influence of dextrin and sucrose on growth and dermatitis. J. Nutrition, vol. 11, p. 391.

Bender, R. C., and G. E. Supplee 1937 Significance of quantitative relationships in vitamin B complex studies. J. Am. Chem. Soc., vol. 59, p. 1178.

Birch, T. W. 1938 The relation between vitamin B_6 and the unsaturated fatty acid factor. J. Biol. Chem., vol. 124, p. 775.

BIRCH, T. W., AND P. GYÖRGY 1936 A study of the chemical nature of vitamin B_6 and methods for its preparation in a concentrated state. Biochem. J., vol. 30, p. 304.

BROWN, J. B., AND J. FRANKEL 1938 Studies on the chemistry of the fatty acids. J. Am. Chem. Soc., vol. 60, p. 54.

BROWN, J. B., AND G. G. STONER 1937 Studies on the chemistry of the fatty acids. J. Am. Chem. Soc., vol. 59, p. 3.

BROWN, W. R., AND G. O. BURR 1936 Some recent studies on fat deficiency. J. Biol. Chem., vol. 114, p. XVI.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

———— 1930 On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem., vol. 86, p. 587.

GYÖRGY, PAUL 1935 Investigations on the vitamin B_2 complex. I. The differentiation of lactoflavin and the 'rat antipellagra' factor. Biochem. J., vol. 29, p. 741.

HALLIDAY, N., AND H. M. EVANS 1937 On the fractionation of the vitamin B_2 complex from various source materials. J. Biol. Chem., vol. 118, p. 255.

HOGAN, A. G., AND L. R. RICHARDSON 1934 Irradiated vitamin B complex and dermatitis. J. Nutrition, vol. 8, p. 385.

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, p. 557.

QUACKENBUSH, F. W., AND H. STEENBOCK 1938 Acrodynia and the essential fatty acids. J. Biol. Chem., vol. 123, p. xcvii.

RICHARDSON, L. R., AND A. G. HOGAN 1936 Skin lesions of the rat associated with the vitamin B complex. Mo. Agr. Exp. Sta. Res. Bull. 241.

ROLLETT, A. 1909 Zur kenntnis der Linolsäure. Z. Physiol. Chem., vol. 62, p. 410.

SALMON, W. D. 1938 The effect of certain oils in alleviating localized erythematous dermatitis (acrodynia or vitamin B_6 deficiency) in rats. J. Biol. Chem., vol. 123, p. civ.

STEENBOCK, H. 1923 A satisfactory ration for stock rats. Science, vol. 58, p. 449.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, vol. 75, p. 339.

STUDIES OF FAT-FREE DIETS

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Evans and Burr ('27, '27 a) demonstrated the existence of a deficiency syndrome in the rat produced by the exclusion of fat from the diet. The syndrome was characterized by subnormal weight and poor reproductive performance. Subsequent work by Burr and co-workers ('29, '32) showed that certain fatty acids, linoleic and linolenic, were curative of and prophylactic for this deficiency disease. Turpeinen ('37, '38) has recently suggested that the animal organism might primarily require arachidonic acid rather than linoleic or linolenic acid. His suggestion was based on the demonstration that arachidonic acid was curative at a level of 33 mg., whereas 100 mg. of methyl linoleate was necessary to produce an equivalent optimal growth in plateaued fat-deficient animals. Burr, Burr and Miller ('32) had stated that the maximum growth effect of linoleic acid was reached at a 10-drop level per rat per day; however, Bull ('37) quotes Burr to the effect that ½ drop of linoleate daily is a dosage sufficient to cure scaliness of feet, tail and skin, and to restore growth to a fat-deficient rat. This paper deals with controversial points in the study of fat-free diets.

SECTION I. FAT DEFICIENCY SYNDROME

Procedure

The basal fat-free diet used in these experiments had the following composition:

	%
Casein (alcohol- and ether-extracted)	20.0
Northwestern dried yeast (ether-extracted)	10.0
Sucrose	64.0
Salt mixture no. 51	6.0

Salt mixture no. 51

$CaCO_3$	1.5
KCl	1.0
NaCl	0.5
$NaHCO_3$	0.7
MgO	0.2
Fe citrate	0.5
KH_2PO_4	1.7
	6.1

The ether extractions of casein and yeast were made in Soxhlet apparatus for 24-hour periods. The casein had previously been extracted by first cold 70% and then cold 95% alcohol for a like period of time. Ether extraction over a period beyond 24 hours proved valueless as no additional lipoid material was removed.

One group of animals, seventy in number, was maintained on this basal fat-free diet from weaning with the following supplements:

Carotene[1] (dissolved in ether)	0.08 mg. per rat per day
Ergosterol[1] (irradiated) (dissolved in ether)	100 international units per rat per day
Vitamin E (non-saponifiable matter of wheat germ oil)	≎ 3 gm. wheat germ oil per week

This constituted diet A.

A second group received 10 drops of methyl linoleate from weaning time, the purpose being to compare the growth response made by rats on this diet when linoleate was used prophylactically. Twenty-two rats were placed on this diet.

[1] Supplied through the courtesy of Dr. Charles E. Bills, Mead Johnson and Company.

The supplements were as follows:

Carotene (dissolved in ethyl laurate)	0.08 mg. per rat per day
Ergosterol (irradiated; dissolved in ethyl oleate)	100 international units per rat per day
Vitamin E (non-saponifiable matter of wheat germ oil)	≎ 3 gm. wheat germ oil per week
Methyl linoleate	10 drops or 300 mg. per rat per day

This group of supplements, plus the basal fat-free diet, constitutes diet B.

Methyl linoleate was prepared by esterifying a commercial product of linoleic acid. This product was secured from the Glyco Company and is prepared from linseed oil. The esterification was the methyl alcohol-anhydrous hydrogen chloride technic. Fractionation in a 6-foot, high vacuum, laboratory fractionating column gave a product of iodine number 150.3. The preparation contains both methyl linolenate and methyl oleate, and is referred to as crude methyl linoleate.

For the more refined feeding tests reported herein, a methyl linoleate was prepared by the bromination procedure of Rollet ('09). The methyl linolenate used was also prepared by the method of Rollet ('09 a).

Iodine numbers	*Observed*	*Theoretical*
Crude linoleate	150.3	
Pure linoleate	173.1	181.2
Pure linolenate	259.3	273.7

Even on this diet containing every known essential, the full adult size is not attained. After the growth curves have flattened for several weeks the rats again grow promptly when given our stock diet. Stimulation of a new growth increment was the principal criterion used in the study of certain additions to the diet.

RESULTS

Symptomatology

Burr and Burr ('29) reported caudal necrosis as the most marked symptom in their fat deficiency disease. Scaly feet, dandruff, alopecia and hematuria were listed as other criteria of the existence of an unsaturated fatty acid deficiency. A year later, Burr and Burr ('30) stated that caudal necrosis does not occur in the first 4 to 6 months on the diet, and stressed scaliness of the feet as the most marked manifestation of the disease. Brown and Burr ('36) later observed that scaliness of the feet varied with humidity, being less severe in summer and more severe in winter. Evans and Lepkovsky ('32) observed no caudal necrosis in their animals, and they question scaliness of the feet as a specific symptom because it occurred in animals receiving a diet which was not fat free.

TABLE 1

Comparison of growth (in grams) of rats on fat-free basal diet. Curative and prophylactic values

		AVERAGE PLATEAU WEIGHT	AVERAGE FINAL WEIGHT	MAXIMUM WEIGHT	MINIMUM WEIGHT	NUMBER OF RATS
Fat-free	♂	205	165	234	137	35
	♀	171	134	177	117	35
Prophylactic dose, 10 drops of crude linoleate	♂	281	...[1]	311	251	15
	♀	182	...[1]	202	146	15
Prophylactic dose, 3 drops of crude linoleate[2]	♂	260	...[1]	287	220	25
	♀	190	...[1]	215	160	25
Curative dose, 10 drops of crude linoleate	♂	266	..[1]	298	204	25
	♀	173	...[1]	205	129	25
Stock animals	♂	360	...[1]	425	320	Colony
	♀	220	...[1]	280	200	Colony
Burr's figures ('29)	♂	170		270[3]		
	♀	150				

[1] Maintained weight at plateau level.

[2] In Burr's experiment, lard was added to the diet.

[3] Values from a former unpublished series.

In this series of seventy animals on a fat-free diet, diet A, approximately 20% showed caudal necrosis within 6 months. The remainder showed no macroscopic evidence of caudal involvement. Hematuria was observed in 50% of the animals, indicating gross renal damage. The two apparently infallible symptoms of the deficiency were subnormal weight and scaliness of the feet. Conditions of humidity were not studied. Alopecia was observed but in only a small percentage of cases.

The disagreement among previous observers doubtless can be explained on a basis of biological variations. This factor is indicated by differences in growth behavior as shown in this paper.

Growth behavior on fat-free diets

Table 1 is self-explanatory. It is seen that the maximum average weight of rats fed methyl linoleate prophylactically does not exceed to any significant degree the values for rats fed methyl linoleate curatively; thus indicating, if growth is used as a criterion, the fact that the fat-free regime does not permanently damage the rat under the experimental conditions observed. Several rats when fed linoleate after a 1- or 2-month period of decline in weight failed to respond. The great biological variation shown by the rats is worthy of note.

TABLE 2

Response to linoleate therapy—10 drops per day level of methyl linoleate

	RESPONSE IN 6 WEEKS		RESPONSE IN 14 WEEKS	
	♂	♀	♂	♀
	gm.	*gm.*	*gm.*	*gm.*
Average	46	23	81	31
Maximum	73	44	142	49
Minimum	15	6	47	14

Purified linoleate used during first 4 weeks; crude preparation used subsequently. There were twenty-five rats in each group.

Curves of the animals on a fat-free diet, diet A, reached a plateau in 4 to 5 months. Those for rats on diets supplemented with linoleic acid reached a second plateau in 6 to 12 months. For animals fed linoleic acid prophylactically, a plateau was reached in 7 to 9 months.

The values for animals recorded in table 2 show evidences again of a great biological variation. In the case of one male, a growth response of 200% was elicited; the animal's weight increased to double the level of the plateau reached on the fat-free diet. Variations between litters are observed.

It is to be noted from table 3 that the rate of response on the various dosage levels does not differ materially. In order

TABLE 3

Response to various dosages of methyl linoleate. The weights are measured in grams

DOSAGE	1 DROP		2 DROPS		3 DROPS		5 DROPS	8 DROPS		10 DROPS		12 DROPS	
NUMBER AND SEX OF RATS	2 ♂	2 ♀	2 ♂	2 ♀	2 ♂	2 ♀	2 ♂	2 ♂	2 ♀	2 ♂	2 ♀	2 ♂	2 ♀
Average response in 4 weeks	21	12	28	13	17	30	13	26	5	25	10	13	9
Average response in 8 weeks	38	30	48	27	32	29	27	53	34	41	23	21	12
Average response in 12 weeks	51	32	67	33	41	42	53	75	35	49	30	46	21
Total gain on indicated dose	51	32	69	35	48	45	53	77	35	50	30	48	23
Average response on 10-drop level of linoleate after plateau on indicated dosage	0 in 5 weeks	0 in 4 weeks	+7 in 5 weeks	0 in 6 weeks	2 in 6 weeks	+6 in 5 weeks	0 in 6 weeks	0 in 6 weeks	0 in 6 weeks	—1 in 4 weeks	—3 in 4 weeks	+3 in 5 weeks	—7 in 5 weeks

The linoleate fed during the first 4 weeks was prepared by the method of Rollett ('09) and subsequently our crude preparation of linoleic methyl ester was used. The rats used on the various linoleate levels had reached stationary weights at the same age.

to eliminate toxicity as a factor in this experiment, the level of methyl linoleate was doubled, 20 drops per day, in the diet of two rats, and no indications of toxicity, as shown by weight loss occurred in a period of 4 weeks. Also, to check the results for these two animals, the methyl linoleate was removed from the diet of two rats. A failure of response was taken as indicative of the absence of toxicity in the methyl linoleate as prepared and in the amount administered.

The rate of response to linoleate therapy as indicated in table 3 does not vary with dosage, but seems constant for dosages from 30 to 360 mg. of methyl linoleate daily. The total responses do not differ significantly. The lack of response to a 10-drop level of methyl linoleate after a plateau had been reached on the lower dosages (30 to 240 mg.) indicates that the optimal dosage of methyl linoleate lies somewhere below 1 drop per day per rat of the preparation used.

Six rats on the basal diet including methyl linoleate at a 10-drop level, plus all known vitamins whose curves had reached a plateau, were then given an additional supplement of a 4-drop level of methyl linolenate. The methyl linoleate used from weaning was a product prepared from corn oil by the bromination procedure of Rollet ('09). It was shown to be free of methyl linolenate. Five of the rats failed to show any response in 7 weeks; the sixth showed a slight gain. It may be concluded that methyl linolenate cannot supplement methyl linoleate in the diet of a rat. This has been assumed, but not hitherto proved.

Lard was fed at a level of 1 gm. per day per rat to five rats. The response noted was comparable with regard to time, rate of response and total gain shown by rats fed methyl linoleate. It was found impossible to obtain an added response in these rats, whose curves had reached a plateau on lard, by the addition of methyl linoleate. The reverse also held: rats stationary in weight on a diet containing methyl linoleate gave no response to the addition of lard. It is concluded that the effect of lard depends on its content of the essential unsaturated fatty acids. The samples of lard used in this work did not produce the brain or liver effect discussed in section II of this paper. It is to be emphasized that growth responses obtained with lard are in no way superior to those obtained with pure methyl linoleate.

DISCUSSION

The contradictory state of the literature (Burr and Burr, '29, '30; Evans and Lepkovsky, '32) with regard to symptomatology in a fat deficiency disease has been explained on a basis of variation in severity with rise and fall of humidity. Further, it is possible that certain strains of inbred rats will show a difference in symptoms according to their ability to resist depletion of body stores of the essential unsaturated fatty acids. The incidence of caudal necrosis and kidney damage in the series here considered is indicative of this variation. Subnormal weight and scaliness of hind feet are the true and nearly constant indications of a fatty acid deficiency (linoleic or linolenic acid); however, even the incidence of scaly feet is not 100%.

The maximum average weights of rats fed linoleate curatively and prophylactically are comparable, thus indicating the absence of permanent damage as indicated by growth when rats are raised from weaning to plateau weights on fat-free diets. Later in the course of the deficiency disease, when the animals have lost a considerable amount of body weight, it is impossible to elicit responses even with high dosages of linoleate.

The dosages reported in the papers of Burr and Burr ('29, '30) and of Evans and Lepkovsky ('32) would seem to indicate the presence of two factors in the materials with which they were working. The fact that the minimum dosage of linoleic acid for maximum effect probably lies at or below a level of 1 drop (30 mg.) should exclude the possibility of an additional factor other than linoleic or linolenic acid present in lard. Burr's results as presented in Bull's monograph ('37) agree with our findings. However, the optimal dosage level reported by Turpeinen ('37)—100 mg. for methyl linoleate—far exceeds this value. In our experiment, four rats were used on each dosage level. It is to be emphasized that this small number of rats does not permit an accurate statement of the minimal dosage for optimal growth effect if comparison of growth curves is used as the basis. Animals show a great biological variation in growth response. The failure of larger dosages of methyl linoleate to produce growth in animals plateaued on lower dosage levels is the basis of our statement that a minimum dosage for optimal growth effect is at or below 30 mg. per rat per day. No conclusions have been drawn from comparison of growth curves.

The evidence presented by Burr, Burr and Miller ('32) to establish the mutual replaceability of methyl linoleate and linolenate is inconclusive. They compared growth response on a 3-drop level of methyl linoleate and on a mixture of 1½ drops of linoleate plus 1½ drops of linolenate, and finding the responses comparable concluded the mutual replaceability. This evidence is not conclusive, as enough linoleate was present to give the total effect in the absence of linolenate. In order to eliminate the possibility that linolenic acid might supplement linoleic acid, we fed optimal amounts of methyl linoleate and attempted, by the addition of methyl linolenate, to obtain growth response in the animals whose weights had reached a plateau. Lack of response to the linolenic acid dosage indicated that it cannot supplement linoleic acid in the diet of the rat.

SECTION II. GROWTH EFFECT OF NATURAL DIETARY SUPPLEMENTS

From table 1 it is seen that rats fed a diet complete with regard to all known vitamins, including the essential unsaturated fatty acids, do not attain full adult size. It would seem, therefore, that the diet is still lacking in some factor essential for optimal nutrition. It was inferred that this factor would be associated with the fat fraction from natural products, as the inclusion of ether-extracted yeast provided all the water-soluble factors needed by the rat.

In recent years there have been numerous reports of growth factors outside the water-soluble group. Many of these reports seem to have overlooked the work of Evans ('28, '29) on the growth effect of wheat germ oil. Sterol-free vitamin E concentrates showed this same growth-stimulating effect. The conclusion was that the effect was due to the vitamin E content of the materials fed. As indicated previously, vitamin E was provided in our basic diet from weaning, thus definitely excluding this factor as the source of effects observed in further study.

PROCEDURE

The weights of the animals were raised to a plateau on the diets described in section I. A plateau was said to have been reached when in 5 successive weeks the weight of the animal had remained constant or declined. It was felt that the group fed a fat-free diet, diet A, for a time might be depleted of a possible missing fat-soluble factor.

Each of the vitamin supplements was checked to assure optimal feeding levels. Blumberg ('35) has reported the completeness of this diet with respect to all known factors except vitamin E. This fact we confirmed in a series of animals, using Blumberg's technic. Vitamin E as a factor affecting growth has been excluded in this study by feeding the non-saponifiable matter of wheat germ oil equivalent to 3 gm. of the oil per week. After a plateau was reached the quantity was doubled, and, as no growth response was elicited, it was concluded that vitamin E was present in optimal amounts. The vitamin E content relative to the antisterility effect was proved by incorporating the non-saponifiable matter of wheat germ oil, at a level equal to 250 mg. per week of the oil, and noting fertility by mating tests through three successive gestations.

The supplement fed when plateau weights were reached was given at a level of 1 gm. per rat per day in every case, whether oil, fat, liver, brain, brain extract, etc. These materials were added with the regular supplements.

The oils used were pure products obtained from Eimer and Amend. The wheat germ oil was prepared fresh from wheat germ as used. The brain and liver were purchased twice weekly and preserved in a refrigerator.

RESULTS

The animals fed the diet which is complete with regard to all known dietary essentials reached stationary weights materially below the figures for stock animals. For males, the stock figure for average maximum weight is 360 gm., while for the experimental animals this value is 280 gm., approximately 75% of the stock value. For females, the stock colony value is 220 gm., while for the experimental female animals 182 is the average figure. This is 80% of the stock animal value. The weights reached a plateau in from 7 to 10 months.

It is obvious that the synthetic diet used is deficient in one or several respects. To assure the fact that animals raised on this synthetic diet were not permanently stunted, a group of twenty-three rats was placed on the McCollum stock diet. The composition of this diet follows:

Wheat (soft)	20.0
Maize (yellow)	20.0
Rolled oats	20.0
Flaxseed oil meal	10.0
Casein (crude)	3.5
Whole milk powder	25.0
$CaCO_3$	0.5
NaCl	1.0
Fe citrate (50 gm. per 100 pounds)	
$CuSO_4 \cdot 5H_2O$ (20 gm. per 100 pounds)	

Fresh greens—cabbage, kale, spinach, etc., 3 days a week

The response observed indicated the presence in the stock ration of some factor not present in the synthetic diet. From the composition of the stock ration, it seemed worth while to try maize oil, as maize constituted a large portion of this diet. Responses were observed as indicated in table 4. Maize oil was weakly positive. Linseed, soya bean, perilla, poppy seed and wheat germ oils were shown to be entirely negative. This rules out the factor of caloric intake as the possible explanation of the growth responses shown on various positive supplements. Liver proved considerably more potent than maize oil. Brain, however, proved the most potent source investigated, and the only source in which 100% response was observed.

TABLE 4

Response of rats on diet B with various supplements. The weights are measured in grams

SUPPLEMENT	NUMBER OF RATS		AGE LIMITS WHEN GIVEN + SUPPLEMENT IN WEEKS	PLATEAUED WEIGHTS						AVERAGE GAIN IN 4 WEEKS		AVERAGE GAIN IN 8 WEEKS		AVERAGE TOTAL GAIN		MINIMUM TOTAL GAIN		MAXIMUM TOTAL GAIN		AVERAGE FINAL WEIGHT		MAXIMUM FINAL WEIGHT		MINIMUM FINAL WEIGHT	
	♂	♀		Average ♂	Maximum ♂	Minimum ♂	Average ♀	Maximum ♀	Minimum ♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Corn oil	9	7	31 to 45	260	306	235	171	196	147	4	2.5	8	4	16	10	—20	—9	40	26	276	181	336	216	257	155
Liver	3	4	34 to 36	275	291	251	197	201	140	17	5.3	30	9	35	14	21	5	41	24	310	211	318	218	299	164
Stock diet	10	13	28 to 38	269	284	265	167	180	151	29	17	45	26	45	26	22	18	79	39	314	193	353	209	300	173
Brain	13	11	35 to 48	273	299	242	196	205	180	23	16	43	23	45	25	32	18	70	33	319	219	343	230	288	207
Ether extract of brain	3	3	29 to 36	276	286	266	189	195	182	20	15	35	21	35	21	30	16	40	26	311	209	334	216	295	199
			AGE WHEN MADE CONTROLS																						
Controls	7	6	31 to 45	278	311	243	177	196	150	1	0	—7	2	—3	0	—17	—5	3	3	275	177	314	200	236	150

The ether solubility of the factor was demonstrated by extraction of brain in Soxhlet apparatus. The residue showed no activity, while the extract showed full potency. The factor was not water-soluble, as shown by a series of six rats whose diets were supplemented by a water extract of brain. A commercial lecithin showed no potency even when fed at a level of 0.5 gm. daily.

DISCUSSION

The report of Evans ('28, '29) in which he discusses the growth effect of wheat germ oil on vitamin E-free, sterile males seems to have been little considered in nutritional literature up to the last few years. Vitamin E was generally regarded as an antisterility vitamin and not as a growth factor. Several reports (Blumberg, '35; Martin, '37) have appeared which confirm the work of Evans by suggesting growth factors in wheat germ oil and various extracts of wheat germ oil. Evans, Emerson and Emerson ('38) using alpha tocopherol conclusively proved this point.

Numerous workers (Guha, '31; Mapson, '31; Smith and Seegers, '34; Kline, Elvehjem, Keenan and Hart, '34) report the presence in liver of a factor affecting growth. Of these, few have even considered the growth factor, vitamin E.

Work reported to date on this growth factor in liver can be criticized from three standpoints. First, the basal diets used have not been highly purified. Second, vitamin E has never been definitely excluded as a factor in the growth responses observed with liver supplements. Third, prophylactic tests have been used exclusively, fair growth being compared with good growth. The work here reported has eliminated these three possibilities of criticism. The basal diet used is highly purified. Vitamin E is provided by incorporating in the basal diet the non-saponifiable matter of wheat germ oil equivalent to 3 gm. per week per rat of the oil and is therefore excluded as the factor inducing growth response reported in these experiments. The potency of the oil for vitamin E activity was tested and shown to be high. That vitamin E had been included in optimal amounts in the basal diet was demonstrated when we doubled the dosage and observed no weight response in animals which had reached a stationary weight with the amount of the non-saponifiable matter of wheat germ oil mentioned above. The curative method of testing was used. This eliminates the necessity of comparing growth, as we have found that biological variations in animals render inaccurate the use of prophylactic methods.

Turpeinen ('37) reports the superiority of arachidonic to linoleic acid as a curative substance in 'fat deficiency' disease. He suggests that the need of the animal organism is primarily for arachidonic acid. The bearing of this work on the results here reported is problematical. Both liver and brain contain arachidonic acid, and both exhibited the growth promoting effect observed.

The responses observed are not great but are definite. The possibility of obtaining large growth responses in rats approaching a normal for stock rats is very slight in any event. The synthetic basal diet is nearly complete. It is quite possible that the inclusion of the factor herein described in a basal diet would permit growth in rats equal to that obtained on a diet of natural foods.

No claim is made for the existence of á new vitamin. However, it is suggested that one explanation of the results reported in this paper is the existence of another vitamin.

SUMMARY

Data are presented comparing the average maximum growth of rats on fat-free diets with that of rats fed methyl linoleate curatively and prophylactically. The minimum level of methyl linoleate for optimal growth effects has been tentatively placed at or below 1 drop (30 mg.) per day per rat. The inability of methyl linolenate to supplement methyl linoleate is proved.

The observation has been made that rats fed a highly purified synthetic diet, complete in all known dietary essentials, plateau at subnormal weights. These rats show positive growth response to supplements of brain and liver.

LITERATURE CITED

BROWN, W. R., AND G. O. BURR 1936 Some recent studies in fat deficiency. J. Biol. Chem., vol. 114, p. xvi.

BLUMBERG, H. 1935 A growth deficiency disease, curable by wheat germ oil. J. Biol. Chem., vol. 108, p. 227.

BULL, HENRY B. 1937 Biochemistry of the Lipids, p. 26.

BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem., vol. 82, p. 345.

——— 1930 On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem., vol. 86, p. 587.

BURR, G. O., M. M. BURR AND E. S. MILLER 1932 On the fatty acid essential in nutrition. III. J. Biol. Chem., vol. 97, p. 1.

EVANS, H. M. 1928–1929 The relation of vitamin E to growth and vigor. J. Nutrition, vol. 1, p. 23.

EVANS, H. M., AND G. O. BURR 1927 A new dietary deficiency with highly purified diets. Proc. Soc. Exp. Biol. and Med., vol. 24, p. 740.

——— 1927 a The antisterility vitamin fat-soluble E. U. of California, Press, Berkeley.

EVANS, H. M., AND S. LEPKOVSKY 1932 Vital need of the body for certain unsaturated fatty acids. 1. Experiments with fat-free diets in which sucrose furnishes the sole source of energy. J. Biol. Chem., vol. 96, p. 143.

EVANS, H. M., O. H. EMERSON AND G. A. EMERSON 1938 Alpha tocopherol and growth. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 197.

GUHA, B. C. 1931 Observations on certain factors necessary for the normal nutrition of the rat. Biochem. J., vol. 25, p. 960.

KLINE, O. L., C. A. ELVEHJEM, J. A. KEENAN AND E. B. HART 1934 Studies on the growth factor in liver. J. Biol. Chem., vol. 107, p. 107.

MAPSON, LESLIE W. 1932 Evidence of a dietary principle stimulating general growth and lactation. Biochem. J., vol. 26, p. 970.

MARTIN, GUSTAV J. 1937 Vitamin E. J. Nutrition, vol. 13, p. 679.

ROLLET, A. 1909 Zur Kenntnis der Linolsäure. Z. Physiol. Chem., Bd. 62, S. 410.

——— 1909 a Zur Kenntnis der Linolensäure. Z. Physiol. Chem.. Bd. 62, S. 422.

SMITH, H. GREGG, AND WALTER H. SEEGERS 1934 The nutritive value of animal tissues in growth, reproduction and lactation. J. Nutrition, vol. 7, p. 195.

TURPEINEN, OSMO 1937 Effectiveness of arachidonic acid in curing 'fat deficiency' disease. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 37.

——— 1938 Further studies on the unsaturated fatty acids essential in nutrition. J. Nutrition, vol. 15, p. 351.

THE CONCENTRATION OF VITAMIN B_1 IN THE TISSUES OF THE RAT

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The actual function of vitamin B_1 in the animal organism is as yet imperfectly understood. In an earlier paper on vitamin B_1 metabolism (Light, Schultz, Atkin and Cracas, '38) we reported experiments on the excretion of the vitamin. We have continued our studies to determine the effect of the feeding level of vitamin B_1 on its concentration in the tissues of the rat.

Leong ('37) and Harris and Leong ('36) have reported on similar studies, using the bradycardia method of assay. Their work appeared while our studies were in progress, but it was thought advisable for us to continue in view of the difference in methods. The B_1 assay method in this and previous work is the fermentation method of Schultz, Atkin and Frey ('37 a, b, and 38). The studies on excretion of the vitamin indicated that tissue concentration of the vitamin reached a maximum at some point at or above a 65 micrograms intake per day. The results of total rat analysis tend to confirm that conclusion. To determine whether any particular organ played a major function in vitamin retention, analyses of the individual tissues of rats on both high and low vitamin levels were made.

EXPERIMENTAL

In all cases the animals were placed on a B-complex free diet composed of:

	%
Rice starch	62
Vitamin-free casein	18
Hydrogenated cottonseed oil[1]	14
Cod liver oil	2
Salt mixture	4

When indicated the vitamins of the G complex were supplied by a separate supplement of dry yeast. The vitamin B_1[2] content of this yeast (no. Y-462) was 30 micrograms per gram, and this was considered in setting the B_1 levels. Additional B_1, as the crystalline product dissolved in a 20% alcoholic solution, was given as indicated in the tables.

Light et al. ('38) showed that, when animals are continued on a uniform vitamin B_1 intake, they eventually reach a condition called excretion equilibrium, where the vitamin output in the urine remains constant and related to the intake level. In the present work all animals were brought to excretion equilibrium before analyzing. We adopted this as a criterion and thus made certain that the tissues of the animals had been adjusted to the particular feeding level at the time of analysis. In order to minimize differences due to the B_1 unabsorbed from the intestinal tract, the animals were killed 22 hours after receiving the last vitamin B_1 supplement.

TOTAL RAT ANALYSIS

For total rat analysis the carcass, after removal of the liver, was chopped well with a meat cleaver and then minced by passage through a meat grinder. The mass, including all fluids, was extracted three times with boiling acidulated water.

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[1] Crisco.

[2] In this laboratory we have found by the rat growth method that 4 micrograms B_1 is equal to 1 international unit. By eluting the B_1 from the international standard and testing the eluate by the gas method, we also obtain the figure of 4 micrograms B_1 per 10 mg. of international standard. With the rat curative method, on the other hand, we have obtained 2.7 to 3.0 micrograms per unit as the ratio between crystalline B_1 and the international standard adsorbate.

The liver was treated separately in a similar manner. Three extractions in the manner described were found adequate, since two different carcasses, on analysis of the first, second and third extracts, gave 189, 42 and 7.7 micrograms respectively on one carcass, and 46, 20 and 1.5 micrograms on the other. The extracts were combined, filtered, made to volume, and sterilized in flowing steam. Table 1 gives the results obtained by anaysis of extracts so made.

The results show a gradual increase in the B_1 content of the body with increasing levels of intake up to 65 γ. Above this level there does not seem to be any increase. This result is consistent with the observations made in our previous work (Light et al., '38) that no excretion lag could be observed at levels of 65 γ and above. This means that a rat, in excretion equlibrium at 65 γ of B_1 per day, cannot retain any more vitamin and will fall into immediate excretion equilibrium should the intake level be raised. In other words, when excretion equilibrium is reached on a 65 γ daily intake level the body tissues are saturated with respect to the vitamin.

TABLE 1

Effect of vitamin B_1 intake level on total vitamin content of the rat

VITAMIN SUPPLEMENT (PER DAY)			BODY WEIGHT (AV. OF 3 RATS)	MICROGRAMS B_1 PER GRAM BODY WEIGHT	MICROGRAMS B_1 PER GRAM LIVER
Dry yeast no. Y-462	Vitamin B_1	Total B_1			
gm	γ	γ	*gm.*	*Average*	*Average*
0.33	..	10	118	0.54	1.08
0.66	..	20	186	0.49	1.25
0.50	15	30	198	0.92	2.8
0.50	25	40	253	1.23	4.8
0.50	35	50	244	1.34	4.85
0.50	50	65	218	1.77	6.6
0.50	50	65	149	2.14	8.15
0.50	100	115	205	1.63	5.3
0.50	500	515	195	1.88	6.8

Comparison of the data in columns 4 and 5 of table 1 shows that there is a rough inverse relationship between body weight and B_1 concentration. This may be due to a variable proportion of body fat, which on analysis has been found to be relatively low in vitamin concentration.

B_1 CONTENT OF INDIVIDUAL ORGANS AND TISSUES

For the purpose of determining the vitamin content of specific parts of the animal, the rats were anaesthetized and decapitated. The desired organs and tissues were immediately dissected out, weighed and ground in a mortar with sand. The mass so obtained was extracted three times with acidulated water at 100°C. The combined extracts were handled in the same manner as the total rat extracts described above. Table 2 gives the results obtained. The eight animals concerned in this table were of the same age and weight at the beginning of the experimental period. They had received 215 micrograms B_1 daily for 58 days, which insured complete tissue saturation with respect to the vitamins.

TABLE 2

Vitamin B_1 content of organs and tissues

Animals	2 ♂		2 ♂		3 ♂ 1 ♀	
Average weight	122 gm.		245 gm.		259 gm.	
Days on test diet	28		28		28	
B_1 supplement	0		15 γ B_1 (0.5 gm. no. Y-462)		215 γ B_1 (0.5 gm. no. Y-462 + 200 γ B_1	
	B_1 γ/GM.	B_1 TOTAL	B_1 γ/GM.	B_1 TOTAL	B_1 γ/GM.	B_1 TOTAL
Liver	0.26	0.92	1.9	14.6	10.0	87.0
Heart	0.68	0.50	2.6	2.2	7.2	5.9
Testes	1.87	2.1	4.7	11.4	6.6	18.4
Kidney	0.39	0.7	2.1	3.2	6.7	11.5
Muscle (skeletal)	0.14		0.57		1.3	
Blood			0.07		0.35	

As might be expected the general range of tissue concentrations corresponds with the total rat concentrations at the different levels. With the lower levels of B_1 intake, the testes (1.87 to 4.7 γ/gm.) are highest in B_1 concentration. The tissue richest in B_1 at the higher levels is the liver, although all tissues have gained in vitamin content. Additional results on the organs and tissues of saturated rats (dose 215 γ/day) were:

	γ/gm.
Body fat	0.1–0.3
Spleen	2.0–3.0
Thymus	3.0–3.5
Brain	2.5–4.2
Thyroid	4.0–6.0
Mesenteric fat	0.2–0.3

DISCUSSION

A daily dose of 65 micrograms of vitamin B_1 is sufficient to saturate the rat body. Leong ('37) reported that saturation was observed at 30 international units per day. By his conversion factor (2.9 micrograms = 1 international unit) this is equivalent to 87 micrograms. The next lower feeding level which he reported was less than 65 micrograms per day, and this did not produce saturation. Thus there is no disagreement between the results of the two investigations on this point.

Storage of vitamin B_1 in the accepted sense does not take place in the rat body. An animal whose body has been surcharged with the vitamin through the feeding of large amounts for an extended period will survive, when deprived of the vitamin, only slightly longer than another animal which had previously existed on a minimum level. This does not mean that the body content of B_1 cannot be increased, but rather that the increase is transitory and can be maintained only by constant high level feeding. The results show that at feeding levels between 10 micrograms and 65 micrograms per day the body concentration is directly related to the intake level.

It is to be noted, however, that there exists an upper limit beyond which body concentration does not go, although the actual concentration is very low in molarity. There is also unequal distribution among the organs. If we assume that the vitamin enters into a loose combination with some substance in the cell, all of our observations on B_1 retention and excretion may be explained. Such a hypothesis receives support from the coenzyme theory of vitamin B_1 function. The vitamin molecule, probably after being phosphorylated, may be capable of combining with a specific protein or similar body to form an enzyme. Such an enzyme would, by analogy with enzymes like the dehydrogenases, readily undergo dissociation into the apoenzyme and the coenzyme. This is the loose combination mentioned above. Furthermore, it is assumed that the normal rat body will have a limited amount of the specific protein and also that organs of the body will vary according to their function in their content of that protein. The above constitutes an explanation of the maximum vitamin concentration in the body and also of its unequal distribution.

The explanation of observed phenomena is complete if it be true that the enzyme complex dissociation is an equilibrium reaction.

$$\text{Coenzyme}_{B_1} + \text{Apoenzyme} \rightleftharpoons \text{Holoenzyme}$$

If the concentration of the coenzyme is not maintained by continued feeding, then the enzyme complex dissociates and the vitamin is speedily lost to the body through excretion in the urine, or by actual destruction. When, however, the concentration of apoenzyme is high with respect to coenzyme, then the dissociation and disappearance of the complex is retarded and may virtually cease. This is found in rats deprived of the vitamin; even at the moment of death due to vitamin deficiency there is an appreciable amount of the vitamin to be found in the carcass.

The question of optimum vitamin intake is an interesting and important one. We have, so far, two fixed points in the rat's metabolism of vitamin B_1: first, the minimum daily requirement for existence and second, the minimum daily intake necessary to produce body saturation. A third point which is not yet established for the rat, using pure B_1, is that level at which the body will not absorb further vitamin B_1 from the intestine. Such a point exists in man as shown by Schultz, Light and Frey ('38). Of the three points mentioned, the one most likely to correspond to the optimum is the second. At this level (B_1 saturation) it may be said that the physiological system of which vitamin B_1 forms a part will be able to operate at maximum efficiency, at least in so far as this vitamin is concerned. Whether such a condition actually represents the optimum, future work must determine.

The testis is the most interesting single organ which we have studied due to the tenacity with which it retains the vitamin when the animal is depleted; the significance of this is not apparent.

SUMMARY

Feeding crystalline vitamin B_1 to white rats in daily doses of more than 65 micrograms does not produce an increase in body concentration. At maximum retention (through feeding) the B_1 concentration is approximately 2 micrograms per gram of body weight.

Analyses of individual organs show a considerable difference in the concentration of vitamin B_1 therein.

The phenomenon of B_1 retention and depletion is discussed in the light of the enzyme theory of vitamin B_1 function.

LITERATURE CITED

HARRIS, L. J., AND P. C. LEONG 1936 Vitamins in human nutrition. The excretion of vitamin B_1 in human urine and its dependence on the dietary intake. Lancet, vol. 230, p. 886.

LEONG, P. C. 1937 Vitamin B_1 in the animal organism. II. A quantitative study of the metabolism of vitamin B_1 in rats. Biochem. J., vol. 31, p. 373.

LIGHT, R. F., A. S. SCHULTZ, L. ATKIN AND L. J. CRACAS 1938 The excretion of vitamin B_1 in the urine and feces. J. Nutrition, vol. 16, p. 333.

SCHULTZ, A., L. ATKIN AND C. N. FREY 1937 a A fermentation test for vitamin B_1. J. Am. Chem. Soc., vol. 59, p. 948.

———— 1937 b A fermentation test for vitamin B. II. J. Am. Chem. Soc., vol. 59, p. 2457.

———— 1938 Influence of nicotinic acid on the fermentation method for vitamin B_1 determination. J. Am. Chem. Soc., vol. 60, p. 1514.

SCHULTZ, A., R. F. LIGHT AND C. N. FREY 1938 Vitamin B_1 metabolism in man. Excretion of B_1 in urine and feces. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 404.

HAIR GROWTH IN YOUNG ALBINO RATS IN RELATION TO BODY SIZE AND QUANTITY OF FOOD

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The effect of diet on body weight, length, organs, systems and teeth in rats has been studied by Jackson ('15), Winters, Smith and Mendel ('27) and Clarke and Smith ('35). Investigations (Osborne and Mendel, '14; Stewart, '16; Jackson and Stewart, '20; Jackson, '36) have also been made on the ability of rats to resume growth after inanition. Only incidental mention has been made concerning the effect of diet on hair growth.

In previous investigations (Butcher, '34, '36, '37) it has been frequently observed that the second growth of hair appeared externally on the dorsum of some rats as early as the thirty-sixth day of life, while on other rats it did not grow until they were 42 or 43 days old. Upon studying the data of previous investigations, it seemed that the factor which was in all likelihood responsible for this variation in the time of the growth of the second coat of hair was either the size of the animal at weaning time or the kind and quantity of food consumed after weaning. Accordingly, carefully planned experiments were undertaken to learn whether the size of the animal at weaning time or the food consumed after weaning modify not only the time of the second hair growth but also the quality of the hair.

The experiments have sufficed to show that, when the animals are well fed after weaning, the size of the animal (22 to

55 gm.) at weaning time does not alter the time of appearance of the second growth of hair. Hair growth is greatly affected, however, by the amount of food consumed following weaning.

METHODS

To determine if the size of the rat at weaning time was responsible for the variation in the time of growth of the second coat of hair, rats (Wistar) of varying weights were secured on the twenty-second day by limiting the number of individuals in the various litters from five to twelve soon after birth. Following weaning, the young rats were fed only dog chow.[1] The hair was removed from their backs with sodium sulphide when they were 24 to 26 days old in order to observe the time of the second hair growth. When the second growth of hair appeared externally on the dorsum, the age, nose-anus length and body weight were carefully recorded in each instance. After the hair began to grow, its quality was studied frequently.

In studying the effect on hair growth of the amount of food consumed after weaning, only rats weighing 40 gm. or more at weaning time were used. To secure such a weight the number in the litter had to be reduced to five or six soon after birth, and usually individuals were selected so that the litter consisted entirely of males or females. After weaning on the twenty-second day, the animals were isolated and were thereafter fed the dog chow. The chow checkers were finely ground to minimize the quantity of food lost from the feeding cups. One or two animals of each litter served as controls, and they had more food accessible than they consumed. Other individuals of the litter were given various quantities of food. The total quantity fed per day was divided between a feeding in the morning and one in the evening. Careful data were kept on the body weight and length at the time of the appearance of the second growth of hair in all rats, and this is recorded in table 2.

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[1] Purina chow, chemical analysis: protein 23%; fat 5%; fiber 4%; ash 7%; nitrogen-free extract 54%; moisture 7%. Mineral analysis: iron 0.018%; magnesium 0.09%; silica 0.23%; potassium 0.56%; sodium 0.67%; chlorine 0.68%; phosphorus 1.17%; calcium 2.22%; sufficient iodine. Vitamin content: A, 4000 international units per pound; B, 275 Sherman units per pound; C, low; D, 500 international units per pound; E, supplied by wheat germ; G, 300 Sherman units per pound.

The effect of the size of the animal at weaning time on hair growth

As may be seen in table 1, the body weight at weaning time varied from 22 to 55 gm. When the rats were fed an unlimited quantity of food after weaning, the weight of the rat at weaning time had no effect on the time of appearance of the second growth of hair, which usually appeared externally in most rats at the age of 37 days. In two litters, fortunately, the animals ranged in weight from 22 to 34 gm. at weaning time. The second coat of hair grew on the dorsum of these rats about the same time. Body weight at the time of appearance of the second growth of hair, the animals usually being 37 or 38 days old, ranged from 54.5 to 129 gm. Therefore, the body did not have to reach a certain weight before the hair grew.

The time of the second hair growth closely correlates with age but with neither body length nor weight. The coefficient of correlation of body length with the time of growth for the ninety-three animals in table 1 equals —0.04; body weight with the time of growth equals —0.127; body length with body weight equals 0.854. These results on hair growth in rats agree with those on rate of feathering in chicks, since Warren ('25) has found that the vigor of chicks has no influence on the rate of feathering.

The effect of the amount of food on hair growth after weaning

Rats weighing 40 to 47 gm. at weaning time

At the beginning of these experiments, it was found that rats gained very little in body weight when fed only 4 gm. of the finely ground chow per day following weaning. Accordingly, it seemed inadvisable to feed a smaller quantity than 4 gm. lest the physical condition of the rat would be too greatly affected.

TABLE 1

The effects of the size of the animal on the time of appearance of the second growth of hair

NUMBER OF ANIMALS	TWENTY-SECOND DAY WEANING				WHEN SECOND GROWTH OF HAIR APPEARED					
	Range of weight	Average weight	Range in length nose-anus	Average length	Age range	Average age	Range in weight	Average weight	Range in length	Average length
	gm.	gm.	mm.				gm.	gm.		
10 ♀	22.5–29.5	26.5	92–101	96.6	37–39	37.7	54.5–71	66	127–137	133
8 ♂	27.5–29.5	28.6	97–103	101	37–38	37.5	65–79	73	140–145	143
16 ♀	32–38	34.2	103–106	104	36–40	37.5	65–91	80.6	135–143	140
9 ♂	32.5–38.5	35.5	104–108	106	36–39	37.3	71–95	83	140–150	146
16 ♀	41–48	43.6	105–110	106	36–41	37.5	67.5–104.5	90.2	142–150	145
17 ♂	41–48	43.8	108–112	110	36–41	38.6	75.5–129	102.3	143–152	147
6 ♀	50–55	52	115–117	115.8	36–38	37	100–112	104	151–162	156
11 ♂	50–52	51	115–121	117	37–40	38.3	84–122	107	148–163	156

TABLE 2

The effect of the quantity of feed on hair growth in young rats

EXPERIMENT	NUMBER OF ANIMALS	AVERAGE WEIGHT ON 22ND DAY	AVERAGE LENGTH NOSE-ANUS	GRAMS OF FEED DAILY UNTIL HAIR APPEARED						WHEN HAIR APPEARED			
				22–24	25–27	28–30	31–33	34–36	37–	Average amount fed	Average age	Average weight	Average length
		gm.	mm.										
1	7 ♀	41	103	4	4	4	4	4	4[1]	196	72	54.6	132
2	3 ♂	43	108	4	4	4	4	4	4[1]	204	74	57	132
3	3 ♀	53.5	117	4	4	4	4	4	4[1]	224	77	58	133
4	5 ♀	43.6	108	5	5	5	5	5	5[1]	195	62	71.6	139
5	2 ♂	42	108	5	5	5	5	5	5[1]	175	58	69.2	138
6	3 ♂	51	115	5	5	5	5	5	5[1]	85	40	77.5	148
7	3 ♀	43.5	106	4	4	5	5	6	6[1]	120	45	66.6	135
8	3 ♂	52.5	117	4	4	5	5	6	6[1]	90	40	79	148
9	3 ♂	43	107	6	6	6	6	6	6[1]	108	41	70.6	141
10	3 ♀	43	108	6	6	6	6	6	6[1]	102	40	73	142
11	3 ♂	47	111	4	5	6	7	8	9[1]	117	40	95	151
12	3 ♀	45	108	4	5	6	7	8	9	99	38	92	147
13	3 ♂	42	106	5	6	7	8	9	10	105	38	88.3	148
14	4 ♀	41.5	104	5	6	7	8	9	10	105	38	83.2	142
15	9 ♂	44.2	110	Abundance of feed						117.4	37	105	150
16	8 ♀	44	106	Abundance of feed						116.8	37	94	146

[1] The last amount was continued daily until the hair appeared.

The second growth of hair on rats, fed only 4 gm. per day, appeared externally when the rats averaged 73 days in age (experiments 1 and 2, table 2). Growth in no instance occurred before the age of 61 days, and no later in any animal than the eighty-fifth day. The hair appeared unevenly over the dorsum and grew slowly so that bare areas persisted on the backs of the rats. Histological sections of skin taken from the dorsum of underfed animals 50 days old showed that the hair bud of the second hair had not begun to grow. Since the bud becomes active about the thirty-second day in well-fed rats, underfeeding had greatly retarded growth. After activity begins in the hair bud, histological observations show that growth of the hair progresses much more slowly in the underfed rats than it does in well-nourished animals.

Five grams of feed per day, 2 in the morning and 3 in the evening, induced hair growth usually when the rats were 60 days old which is about 13 days sooner than when 4 gm. were fed daily (experiments 4 and 5, table 2). The hair, however, appeared in patches externally on the dorsum, and it was 20 days after the first appearance of the hair before the back was completely covered. In one 43-day-old rat (experiment 5) a small area of hair appeared. No more grew until the age of 56 days. The increase to 5 gm. of food per day resulted in longer and heavier animals before hair growth was induced.

Beginning with 4 gm. and then increasing the diet of rats 1 gm. every 6 days (experiment 7) resulted usually in small areas of growth, 1 cm. in diameter, at the age of 45 days. Hair then appeared slowly over the rest of the back, but for 15 days bare areas could be seen. The slight growth on the forty-fifth day indicated that the cyclic activity of the hair coat was being attempted, but that the stimulus was insufficient to induce hair growth over all of the back.

Six grams of feed per day (experiments 9 and 10) induced hair growth on the dorsum quite similar to the growth obtained in experiment 7, where the quantity was increased every 6 days. The hair appeared in patches on 40- and 41-day-old rats, and regions of the back remained nude as late as the fifty-ninth day of life. Body length was affected relatively less than body weight by the food insufficiency. When the rats were given an unrestricted quantity of food after the first appearance of the hair on the forty-first day, hair growth over the back progressed rather evenly.

In experiments 11, 12, 13 and 14 the food intake was practically sufficient to induce the same body length and hair growth as that on animals allowed all they could eat.

Rats weighing 50 gm. or more at weaning time

When 22-day-old rats weighed 50 gm. or more, and were fed only 4 gm. daily, the second hair appeared at the average age of 77 days (experiment 3, table 2). This was about the same time as when the weight of the rat was 40 gm. at the beginning of the experiments. Fifty-gram rats, receiving 5 gm. of feed per day, produced hair externally at the age of 40 days (experiment 6), which was much earlier than the growth on those rats which weighed 42 gm. at weaning and received 5 gm. of food daily (experiment 5). With an increase of food every 6 days in 50-gm. rats (experiment 8), the hair grew on 39- and 40-day-old rats, just 3 days later than on litter mate controls. The hair appeared rather evenly over the dorsum but its growth afterward was greatly retarded.

Further experiments with larger amounts of food were not conducted on rats weighing 50 gm. at weaning time since the hair on such animals receiving 5 gm. daily had grown almost at the regular time.

In these experiments on the effect of the amount of food on hair growth after weaning it is again shown that there is no correlation between body size and the time of hair growth since the coefficient of correlation of body length with the time of hair growth for the sixty-five animals in table 2 equals —0.76; weight with the time of hair growth equals —0.644. The coefficient of correlation for length with weight, however, is 0.784.

DISCUSSION

By cell proliferation the hair bud is able to produce a hair. For this growth the hair bud must have building materials, stimulation and receptivity. Which of these factors is the most important and which is affected most by the underfeeding presents an interesting problem.

Although the building materials may be present, the hair will not grow without some other factor. This is shown by the cyclic activity of the hair coat (Butcher, '34), and by the present experiments on the effect of size at weaning time on hair growth. The hair grew no sooner on the heavier animals.

In the experiments on underfeeding the hair failed to grow in animals which often reached a greater size than rats in which hair growth had been induced by ample food. Other structures, such as the skeleton, grew in these underfed animals, and building substances were therefore present for growth. Waters ('08) likewise found that the skeleton was less influenced than other parts by inadequate diets, and that it persisted in growth when the growth of the animal as a whole had ceased. Thus it seems quite probable that the retardation of hair growth in these underfed animals is not due to insufficient building substances. Either the stimulus or the receptivity of the hair bud must have been impaired by the underfeeding.

By transplantation experiments (Butcher, '36) it was learned that the hair bud was receptive most of the time. In the present underfeeding experiments hair grew at different times and in patches which would indicate that some hair buds were receptive, and there is every reason to believe that all of them in the dorsum were equally receptive.

Eliminating receptivity and lack of building material as the primary causes for the retardation of hair growth upon underfeeding, impairment of the stimulus would seem to be left as the probable cause. If endocrine glands are responsible for initiating the cyclic activity of the hair coat, they may be altered by the insufficiency of nutrition, and not supply the stimulus for hair growth. Possibly changes in the adrenal may account for the retardation. Vincent and Hollenberg ('20) found that in underfed rats, the adrenal hypertrophies greatly. In my experiments ('37) it was shown that adrenalectomy induced an early second growth of hair in the rat, and that this earlier growth was due to the removal of the adrenal's inhibitory influence on the thyroid. It is possible, therefore, that the great retardation of hair growth in underfed animals is due to a disturbed adrenal-thyroid relationship. This problem is being investigated.

In some instances the quantity of food was sufficient to start hair growth at the regular time. The hair then grew slowly, sparsely, and the quality was poor. Therefore, not only is the time of appearance of the hair affected by inadequate food but also the general growth of the hair.

The question arises whether the retarded effect produced by 4 gm. per day has or has not any permanent effect on hair growth. If the daily amount of 4 gm. is continued, sparseness and difference in quality can be noted. If the animal is given all the food that it can consume after the hair starts to grow there seems to be little effect on the buds or the character of the hair.

CONCLUSIONS

1. The size at weaning time (22 to 55 gm.) does not alter the time of appearance of the second growth of hair in the albino rat, providing they are well fed after weaning.
2. After weaning underfeeding greatly retards the rate of hair growth in the rat. Following the initial appearance, the hair grows unevenly and slowly over the remainder of the back.
3. Impairment of the stimulus which induces hair growth seems to be the most probable cause for the retardation of hair growth upon underfeeding.

LITERATURE CITED

BUTCHER, E. O. 1934 The hair cycles in the albino rat. Anat. Rec., vol. 61, p. 5.

——— 1936 Hair growth on skin transplants in the immature albino rat. Anat. Rec., vol. 64, p. 161.

——— 1937 Hair growth in adrenalectomized, and adrenalectomized thyroxin-treated rats. Am. J. Physiol., vol. 120, p. 427.

CLARKE, M. F., AND A. H. SMITH 1935 Effect of a diet poor in salts upon the growth and composition of the incisors of the rat. Am. J. Physiol., vol. 112, p. 286.

JACKSON, C. M. 1915 Effects of acute and chronic inanition upon the relative weights of the various organs and systems of adult albino rats. Am. J. Anat., vol. 18, p. 75.

——— 1936 Recovery in rats upon refeeding after prolonged suppression of growth by dietary deficiency of protein. Am. J. Anat., vol. 58, p. 179.

JACKSON, C. M., AND C. A. STEWART 1920 The effects of inanition in the young upon ultimate size of the body and of various organs in the albino rat. J. Exp. Zool., vol. 30, p. 97.

OSBORNE, T. B., AND L. B. MENDEL 1914 The suppression of growth and the capacity to grow. J. Biol. Chem., vol. 18, p. 95.

STEWART, C. A. 1916 Growth of the body and of the various organs of young albino rats after inanition for various periods. Biol. Bull., vol. 31, p. 16.

VINCENT, S., AND M. S. HOLLENBERG 1920 Changes in the adrenal bodies and the thyroid resulting from inanition. J. Physiol., vol. 54, p. lxix.

WARREN, D. C. 1925 Inheritance of rate of feathering in poultry. J. Heredity, vol. 16, p. 13.

WATERS, H. J. 1908 The capacity of animals to grow under adverse conditions. Proc. Soc. Promotion Agri. Sci., twenty-ninth annual meeting, p. 71.

WINTERS, J. C., A. H. SMITH AND L. B. MENDEL 1927 The effects of dietary deficiencies on the growth of certain body systems and organs. Am. J. Physiol., vol. 80, p. 576.

A LONG-TIME STUDY OF NITROGEN, CALCIUM AND PHOSPHORUS METABOLISM ON A LOW-PROTEIN DIET[1]

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In human balance experiments, daily fluctuations in excreta, even on a constant diet, have been frequently observed. Martin and Robison ('22), studying the biological value of proteins, reported nitrogen fluctuations amounting to 25% of the mean output on a minimum nitrogen diet, in spite of constant food and fluid intakes and regular habits.

To insure reliability of results in nitrogen balance experiments, Boas-Fixsen ('35) emphasized among other things the necessity of a sufficiently long pre-period on the diet to be studied and a long experimental period.

In contrast to this idea, Pyle and Huff ('36) found in calcium and phosphorus studies of pregnant women, that 1-day balances following 2-day pre-periods did not differ significantly from results of Macy et al. ('30) and Coons and Schiefelbusch ('34) with longer periods. Porter-Levin's ('33) findings with pre-school children indicated that 15 to 21 consecutive days of balance were required to cover the range of variation in retention of nitrogen, calcium and phosphorus. Hawks et al. ('37) also working with pre-school children, used 10- to 12-day preliminary periods and collection periods of 21 to 24 days, believing this time was sufficient to show "trends in the metabolism and the 3-day period variations."

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[1] Contribution no. 81.

A tendency to favor long-time balance experiments is evident in these and other studies. Hummel et al. ('36) in their studies on pregnant women say, "It has become increasingly apparent that it is expedient to extend the short balance to a longer period of time . . . ."

Believing that a cyclic retention of nitrogen, calcium and phosphorus might be indicated in a long-time study of women, a 45-day balance experiment was planned in which three young women, 23 and 24 years of age, served as subjects.

METHODS

The three subjects, M, B and A, weighing 49.4, 50.1 and 55.9 kg., respectively, were normal as determined by physical examinations. They were fairly active and were all engaged in graduate study involving similar activities and interests.

A moderately low-protein diet (table 1) was planned to provide 45 Calories per kilogram and to supply 75% of the protein requirement of each subject. Meat was to furnish 85% of this protein. During a 3-day pre-period on the diet the desired distilled water intake was set by each subject at 1100 cc. and the NaCl, at 5.0 gm. daily. The subjects ate the diet for fifteen consecutive 3-day periods.

With the exception of butter, sufficient quantities of the foods to last throughout the diet were purchased at one time and held at suitable temperatures. Butter was churned in two lots and stored below 0°C. The meat, U. S. good beef round,[2] was freed from visible fat, ground three times with a coarse knife, thoroughly mixed and analyzed for nitrogen. On the basis of this analysis the amount of meat needed for the entire study by each subject to supply the desired daily nitrogen was determined. These amounts were then divided into two daily portions of equal weight for each subject and stored below 0°C. until used. The fruit punch, consisting of 150 gm. orange juice and 350 gm. grape juice for a subject, was prepared daily. Sufficient orange juice for the day's needs was expressed, strained and aliquot portions weighed.

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[2] Purchased from the department of animal husbandry of the Kansas State College.

The necessary amount of grape juice was opened, mixed and weighed portions combined with the orange juice. Sucrose and lactose were added in varying amounts for the different subjects in order to adjust the Calories to individual needs. Cabbage sufficient for 3 days was shredded on the first day of a period, stored in an ice box in tightly closed jars, and portions weighed daily as needed. Subjects M and B used a salad dressing of corn oil and dilute citric acid with the cabbage. Subject A disliked oil so increased butterfat and sugar for the necessary Calories. A 'cracker' containing agar, and practically nitrogen-free was made according to the recipe of Long and Pittman ('35).

Breakfast consisted of fruit punch, cracker, butterfat and water. Mineral supplements nearly sufficient to meet the Sherman standards ('37) were taken at this time (table 1). The noon and evening meals consisted of the meat, cabbage salad, fruit punch, cracker and butterfat. The frozen meat for a single meal was allowed to come to room temperature, then baked at 350°F. in individual covered casseroles with a little distilled water and half the subject's daily supply of salt. The meat was served in the casserole which the subject afterward rinsed carefully with a portion of her distilled water to minimize loss.

The daily aliquots of punch were mixed and canned at the end of each period for subsequent analysis. Representative portions of cabbage for a period were mixed, dried in an oven at 80°C., then powdered and stored until analyzed. A sample of meat was preserved in the same way as the cabbage. Crackers, salad oil and filtered butterfat were not analyzed as they were thought too low in nitrogen and minerals to affect results.

Excreta were analyzed by periods. The 24-hour urine was collected under toluol and chilled until the day's collection was complete. It was then mixed and an aliquot for each day's total volume, acidified with HCl, retained for the period-composite. Total urinary acid and pH were determined at the end of each 24-hour day. Carmine was used to mark the

TABLE 1

Daily food intakes on a low-protein diet[1]

SUBJECT	COMPOSITION OF DIET	BEEF ROUND	BEVERAGE	CRACKER	BUTTER FAT	CABBAGE[2] SALAD	$FeC_6H_5O_7 \cdot 3H_2O$	KH_2PO_4	$CaH_4(PO_4)_2 \cdot H_2O$	NaCl	TOTAL
		gm.	*gm.*	*gm*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
M	Weight	92.4	600.0[3]	198.2	54.0	102.1	0.0463	0.548	2.082	5.000	
(49.4 kg.)	Nitrogen	3.24	0.42			0.19					3.85
	Calcium	0.005	0.059			0.038			0.331		0.433
	Phosphorus	0.177	0.067			0.023		0.125	0.512		0.904
	Fiber			2.7		0.8					3.5
	Ml. excess N. acid	12.85						8.05	33.00		53.90
	Ml. excess N. base		22.07			3.45	0.46				25.98
B	Weight	96.6	600.0[3]	198.2	53.5	113.1	0.0458	0.548	2.214	5.000	
(50.1 kg)	Nitrogen	3.38	0.42			0.19					3.99
	Calcium	0.006	0.059			0.038			0.352		0.455
	Phosphorus	0.185	0.067			0.023		0.125	0.545		0.945
	Fiber			2.7		0.8					3.5
	Ml. excess N. acid	13.43						8.05	35.10		56.58
	Ml. excess N. base		22.07			3.45	0.46				25.98
A	Weight	102.5	620.0[3]	297.0	59.0	79.4	0.0449	0.526	2.403	5.000	
(55.9 kg.)	Nitrogen	3.57	0.42			0.19					4.18
	Calcium	0.006	0.059			0.038			0.382		0.485
	Phosphorus	0.196	0.066			0.023		0.120	0.591		0.996
	Fiber			4.0		0.8					4.8
	Ml. excess N. acid	14.26						7.72	48.10		70.08
	Ml. excess N. base		22.07			3.45	0.46				25.98

[1] Based on analysis of foods by periods.

[2] All subjects had 79.4 gm. shredded cabbage; subject M had 22.2 gm. corn oil and 0.7 gm. citric acid; subject B had 33.0 gm. oil and 0.7 gm. citric acid.

[3] 150 gm. strained orange juice, 350 gm. commercial grape juice, 50 gm. lactose, 50 gm. sucrose.

[4] Same as for subject M except 65 gm. lactose and 55 gm. sucrose.

fecal periods. The combined feces for a period were mixed with acidified alcohol, dried below 80°C., then ground.

Analyses of food and excreta were made in triplicate. Nitrogen was determined by the Gunning-Arnold modification of the Kjeldahl procedure: calcium, volumetrically by a method used in the Kansas Agricultural Experiment Station; and phosphorus, gravimetrically by the Neumann method ('02–'03) as modified by Lundell and Hoffman ('23) and McCandless and Burton ('24). Total acid was determined by the Folin method (Hawk and Bergeim, '31) and pH by means of a quinhydrone electrode according to Billmann and Tovborg-Jensen ('27).

RESULTS

The subjects remained in good health and spirits throughout the experiment, doubtless aided to a considerable extent by their interest in the study. The caloric intake was sufficient to maintain weight in all cases. The cracker was the least palatable item in the diet but no serious complaint was voiced. Analysis of the food items showed but little variation in the nitrogen, calcium and phosphorus intakes for the different periods so the diet was regarded as practically constant.

Nitrogen findings. As the meat had been analyzed for nitrogen before the diets were calculated and the other foods contained approximately the amounts of nitrogen estimated, the food actually supplied 75% of the protein requirement for each subject (0.42 gm. per kilogram) with 84.8% of the total nitrogen furnished by meat.

The average daily nitrogen intakes (table 2) were 3.85, 3.99 and 4.18 gm. for subjects M, B and A, respectively, or expressed in milligrams per kilogram, 77.9, 79.6 and 74.8 with standard deviations on the latter basis of only 0.71, 0.70 and 0.72 mg. The maximum variation in intake of nitrogen during the fifteen periods was 2.4 mg. per kilogram for subject M and the minimum was 2.2 mg. for subject A.

The mean dry weight of the feces was 305.8 mg. per kilogram per day. Of this amount, 16.7 mg. were nitrogen which was 21.6% of the total mean nitrogen intake (77.4 mg. per kilogram per day), making the average apparent coefficient of digestibility for the nitrogen 78.4% for the three subjects during the study. The range in digestibility was from a mean of 77.9 to 78.8% for the various subjects although variations from period to period ranged from a low of 68.4% for subject B in period 2 to a high of 88.1% in period 10 for subject A. The average nitrogen absorption for subjects M, B and A was 61.1, 62.1 and 60.0 mg. per kilogram.

TABLE 2

Average daily nitrogen, calcium and phosphorus balances on a low-protein diet

SUBJECT	AVERAGE DAILY INTAKE		AVERAGE DAILY OUTPUT			BALANCE	
	Meat	Total	Urine	Feces	Total	Total	PER KILOGRAM
				Nitrogen			
	gm	*gm.*	*gm*	*gm*	*gm.*	*gm.*	*mg*
M	3.24	3.85	2.48	0.83	3.31	+0.54	+10.9
B	3.38	3.99	2.92	0.88	3.80	+0.19	+ 3.8
A	3.57	4.18	2.83	0.87	3.70	+0.48	+ 8.6
Mean	3.40	4.01	2.74	0.86	3.60	+0.40	+ 7.8
				Calcium			
M	0.005	0.433	0.110	0.377	0.487	−0.054	− 1.1
B	0.006	0.455	0.154	0.400	0.554	−0.099	− 2.0
A	0.006	0.485	0.105	0.470	0.575	−0.090	− 1.6
Mean	0.006	0.458	0.123	0.416	0.539	−0.081	− 1.6
				Phosphorus			
M	0.177	0.904	0.571	0.328	0.899	+0.005	+ 0.1
B	0.185	0.945	0.644	0.360	1.004	−0.059	− 1.2
A	0.196	0.996	0.701	0.322	1.023	−0.027	− 0.5
Mean	0.186	0.948	0.639	0.337	0.975	−0.027	− 0.5

Individual differences were noted in fecal nitrogen. It was most constant for subject B varying only from 55.1 to 58.7 mg. per gram dry feces for any given period, a difference of 3.6 mg. per gram. Subject M showed most variation ranging from 38.6 to 57.7 mg. per gram. The average was 54.9 mg. per gram for the three subjects with A and B exceeding the average and M falling below it.

Urinary nitrogen averaged 68.3% of the nitrogen intake varying from a mean of 73.2% for subject B to 64.4% for subject M. The mean nitrogen output in urine and feces totaled 70 mg. per kilogram, an average of 90% of the intakes of the three subjects.

The nitrogen balances indicating body retention (table 2) show a mean for all subjects of +0.40 gm. per day or 7.8 mg. per kilogram. Subjects M and A were in positive balance throughout the fifteen periods (table 3). Subject B was in decidedly negative balance in periods 1 and 2 and slightly so in periods 6 and 7. Her average for the fifteen periods was close to equilibrium with +0.19 gm. per day or a retention of 3.8 mg. per kilogram which was 4.8% of her nitrogen intake. In contrast, subject M retained 0.54 gm. per day or 10.9 mg. per kilogram which was 14.0% of her intake. Subject A fell between, with a mean retention of +0.48 gm. nitrogen daily or 8.6 mg. per kilogram which amounted to 11.5% of her intake.

TABLE 3

Average daily nitrogen, calcium and phosphorus retentions per kilogram on a low-protein diet

PERIOD	SUBJECT M			SUBJECT B			SUBJECT A		
	N	Ca	P	N	Ca	P	N	Ca	P
	mg	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	7.3	0.7	1.6	—13.1	— 3.3	—2.6	±0.0	— 4.3	—3.3
2	16.4	— 1.6	±0.0	— 7.0	— 3.5	—4.5	5.3	— 1.4	—1.1
3	19.3	— 0.3	—0.3	3.8	0.3	0.5	8.1	— 2.2	—1.1
4	14.2	— 1.9	—0.1	2.2	— 1.8	—2.4	9.2	0.9	1.0
5	16.0	1.1	2.2	13.7	— 1.7	—1.4	4.4	— 2.5	—0.8
6	6.6	1.6	2.0	— 2.0	— 1.0	—2.4	8.5	— 1.3	—0.2
7	4.1	— 3.1	—1.5	— 1.8	— 4.2	—4.6	10.5	— 3.5	—1.2
8	5.0	— 1.4	—0.1	3.3	— 2.1	—0.6	12.7	— 2.7	—0.1
9	12.0	— 4.5	—3.0	8.8	— 3.4	—2.6	0.3	— 3.2	—0.8
10	12.9	— 1.2	—0.8	17.1	— 1.3	2.1	13.2	1.5	2.0
11	4.6	— 2.1	—1.9	± 0.0	— 3.4	—2.2	10.9	0.5	—0.5
12	16.6	0.9	1.3	4.9	— 1.6	0.5	15.7	— 1.2	0.6
13	8.0	— 3.2	0.8	10.0	0.2	1.9	15.9	— 0.6	0.2
14	12.5	— 1.0	—0.5	5.6	— 2.4	—0.3	10.7	— 3.1	0.1
15	7.7	— 0.4	1.6	12.2	— 0.4	0.7	4.3	— 0.3	—2.5
Mean	10.9	— 1.1	0.1	3.8	— 2.0	—1.2	8.6	— 1.6	—0.5
				Per cent of intake					
Mean	14.0	—12.5	0.5	4.8	—22.0	—6.4	11.5	—18.4	—2.8

The mean nitrogen retention on this low-protein diet of 7.8 mg. per kilogram for these young adult women was surprisingly high, approximating one-fourth of that reported by Hawks et al. ('38) for pre-school children on a medium-protein diet. This high retention does not appear to be explained as the result of a nitrogen-poor condition of the subjects previous to the study, for storage tended to increase as the study proceeded. It is also surprising that there was no tendency in these presumably mature subjects to approach nitrogen equilibrium in 45 days on a constant diet.

The average daily calcium intakes were 0.433, 0.455 and 0.485 gm. (table 2) for subjects M, B and A, respectively, which amounted to 8.8, 9.1 and 8.7 mg. per kilogram with standard deviations of 0.20, 0.19 and 0.19 mg. The maximum variation in calcium intake was 0.61 mg. per kilogram for subjects M and A and the minimum was 0.58 mg. for subject B. On the whole the calcium intakes were regarded as relatively constant.

The mean calcium output was 0.539 gm. per day or 10.4 mg. per kilogram. The average daily fecal output was 8.0 mg. per kilogram which was 77.0% of the total output. Subject A, with a more bulky diet, excreted 8.4 mg. calcium per kilogram by way of the feces, somewhat more than the other subjects.

Urinary calcium for the three subjects averaged 2.4 mg. per kilogram. Subject B excreted 3.1 mg. calcium through the urine while subject A, with more bulk, combined a relatively low urinary calcium (1.9 mg. per kilogram) with high fecal calcium.

The average calcium retention was negative for all subjects, the mean for the group being —1.6 mg. per kilogram or —0.081 gm. per day. Subject B was most negative averaging —2.0 mg. per kilogram per day (table 3). Subjects B and A were in positive calcium balance for only two and subject M for three of the fifteen periods of the study.

The average daily phosphorus intakes for the three subjects M, B and A were 0.904, 0.945 and 0.996 gm. per day (table 2) or 18.3, 18.9 and 17.8 mg. per kilogram, respectively, and averaged 18.3 mg. per kilogram. These intakes were remarkably constant showing standard deviations of only 0.24, 0.24 and 0.19 mg. The average fecal phosphorus constituted 35.6% of the intake or 6.5 mg. per kilogram and ranged from a low of 5.8 mg. per kilogram for subject A to a high of 7.2 for subject B.

For subjects M, B and A the urinary phosphorus was 11.6 12.8 and 12.5 mg. per kilogram and averaged 12.3 mg. or 67.4% of the intake. These results are in line with other findings. Porter-Levin ('33) reported a slightly larger portion of total phosphorus excretion in the urine and somewhat smaller amount in the feces in the 2-, 4- and 5-year-old children she observed. Davis ('35), studying older children, reported averages of 42.7% of the total phosphorus excretion in the feces and 57.3% in the urine on an acid diet.

Shohl and Sato ('23) in a study of two infants reported excretion of phosphorus largely by way of the urine which in normal periods averaged 68.5 to 65.0%. They also found that addition of acid shifted phosphorus excretion from stool to urine. Meyers and Fine ('19) in a study of adults reported two-thirds of the phosphorus in the urine and one-third in the feces. The total daily excretion of phosphorus for subjects M, B and A amounted to 18.2, 20.0 and 18.3 mg. per kilogram, averaging 18.8 mg. and resulting in a mean balance for all subjects throughout the study of —0.5 mg. per kilogram. The average daily retention for subjects M, B and A was +0.1, —1.2 and —0.5 mg. per kilogram and the number of negative balance periods were 9, 10 and 10 (table 3), respectively, indicating that the phosphorus was not well utilized at this level. The range of phosphorus retention for the three subjects was from +2.2 mg. per kilogram to —3.0 for subject M, +2.1 to —4.6 for subject B, and +2.0 to —3.3 for subject A.

The diet was acid-forming, having a daily excess of 27.9, 30.6 and 44.1 ml. for N acid for subjects M, B and A. However, the reaction of the diet probably was not sufficiently different from an ordinary one to influence calcium and phosphorus partition greatly.

There was considerable variation in total urinary acid from period to period for each subject and the average excretions differed. The total urinary excretion for subject M was 141.4 ml. of N/10 acid whereas subject B excreted 225.3 ml. and A, 235.3 ml. Some fluctuation was also noted in the urinary pH readings and a slight tendency toward increased acidity throughout the 45 days whereas no change in trend of total acid excretion could be observed. The diet in this experiment contained 350 gm. grape juice and 150 gm. orange juice. Clouse ('35) found that 500 gm. of either grape or orange juice reduced total acid excretion but that grape juice had a less marked effect. She also found a rise in urinary pH with this quantity of orange juice but observed no change when grape juice was used.

The lactose in the diet was probably not a factor in the usage of the calcium and phosphorus. The subjects were not in a growing stage and the quantities used were relatively small.

Six menstrual periods occurred during the study, two for each subject, but the menstrua were not collected. During these periods nitrogen and calcium appeared to be retained in amounts above average in four instances and below in one. Phosphorus was retained in amounts above average in only half of the periods, the other half being below the mean. The unfavorable retention periods were distributed among the three subjects, no one being particularly adversely affected. In no case were the extreme-low points of the study reached at this time.

Basal metabolism tests were made weekly on each subject. The averages of the six tests, expressed in terms of the Aub-DuBois standard as modified by Boothby and Sandiford, were +3.34, —6.72 and —12.42 for subjects M, B and A. No abnormalities were apparent although subject M's may be regarded as rather high. The basal rates showed no consistent relation to menstruation, which agrees with Blunt and Dye ('21). They were high during both periods for subject M at this time and either average or below for the others.

The average per capita intake of fiber, chiefly in the form of agar, was 3.9 gm. daily (table 1). Subjects M and B each consumed 3.5 gm. and A, 4.8 gm., 37% more than the other subjects. In spite of higher fiber intake, subject A had a smaller volume of dry feces with an intermediate value for nitrogen per gram and the lowest fecal nitrogen expressed as milligram per kilogram. Fecal calcium was high and urinary calcium low for A in comparison with the other subjects, although total calcium output was similar. In contrast to the results with calcium, fecal phosphorus was low but total phosphorus output was also close to the average for the three subjects. As the fiber content of all three diets was low, it is doubtful if in any case it had an important influence on the results.

CONCLUSIONS

1. In all subjects considerable variation in retention of nitrogen, calcium and phosphorus was noted from period to period throughout the 45 days of the study. No definite cyclic tendency was apparent.

2. Measurement of the utilization of nitrogen, calcium and phosphorus in young women subjects on moderately low protein diets requires a long-time consecutive balance study, the length of time probably depending on the subject.

3. When meat furnished 85% of the nitrogen of a low-protein diet, the average nitrogen balance for fifteen consecutive 3-day periods was positive for each subject. In two cases there was a trend toward increased nitrogen retention throughout the study although both were presumably adults (23 and 24 years).

4. Levels of 92 and 97% of the commonly accepted standards of 0.68 gm. calcium and 1.32 gm. phosphorus per 70 kg. were not sufficient to prevent losses in the three young women subjects of this study in which protein was fed at 75% of the requirement. The fluctuations in retention of these two minerals tended to parallel, but phosphorus was better used than calcium.

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LITERATURE CITED

BILLMAN, E., AND S. TOVBORG-JENSEN 1927 On the determination of the reaction of soils by means of the quinhydrone electrode. Trans. 2nd Comn. Internatl. Soc. Soil Science B: 236.

BLUNT, K., AND M. DYE 1921 Basal metabolism of normal women. J. Biol. Chem., vol. 47, p. 69.

BOAS-FIXSEN, M. A. 1935 The biological value of protein in nutrition. Nutr. Abst. and Rev., vol. 5, p. 447.

CLOUSE, R. C. 1935 Effect of grape as compared with other fruit juices on urinary acidity and the excretion of organic acids. J. Nutrition, vol. 9, p. 593.

COONS, C. M., AND A. T. SCHIEFELBUSCH 1934 Acid-base balance of the minerals retained during human pregnancy. J. Biol. Chem., vol. 104, p. 757.

DAVIS, N. J. 1935 Calcium, phosphorus and nitrogen retention of children. Effects of acid-forming and base-forming diets. Am. J. Dist. Child., vol. 49, p. 611.

HAWK, P. B., AND O. BERGEIM 1931 Practical Physiological Chemistry. 10th ed. D. Blakiston's Son and Co., Philadelphia, p. 929.

HAWKS, J. E., M. DYE AND M. M. BRAY 1937 An improved technic for metabolism studies in pre-school children with a statistical determination of its reliability. J. Nutrition, vol. 13, p. 51.

HAWKS, J. E., M. M. BRAY AND M. DYE 1938 The influence of diet on the nitrogen balances of pre-school children. J. Nutrition, vol. 15, p. 125.

HUMMEL, F. C., H. R. STERNBERGER, H. A. HUNSCHER AND I. G. MACY 1936 Metabolism of women during the reproductive cycle. VII. Utilization of inorganic elements (a continuous close study of a multipara). J. Nutrition, vol. 11, p. 235.

LONG, Z., AND M. S. PITTMAN 1935 Utilization of meat by human subjects. II. The utilization of the nitrogen and phosphorus of round and liver of beef. J. Nutrition, vol. 9, p. 677.

LUNDELL, G. E F., AND J. I. HOFFMAN 1923 Notes on the determination of phosphorus. J. Ind. and Eng. Chem., vol. 15, p. 44.

MACY, I. G., H. A. HUNSCHER, B. NIMS AND S. S. MCCOSH 1930 Metabolism of women during the reproductive cycle. I. Calcium and phosphorus utilization in pregnancy. J. Biol. Chem., vol. 86, p. 17.

MARTIN, C. J., AND R. ROBISON 1922 The minimum nitrogen expenditure of man and the biological value of various proteins for human nutrition. Biochem. J., vol. 16, p. 407.

McCANDLESS, J. M., AND J. I. BURTON 1924 Sources of error in the determination of phosphoric acid by the molybdate-magnesia method. J. Ind. and Eng. Chem., vol. 16, p. 1267.

MEYERS, V. C., AND M. S. FINE 1919 The relative importance of the intestines and kidneys as excretory channels. Proc. Soc. Exp. Biol. and Med., vol. 16, p. 73.

NEUMANN, A. 1902–1903 Einfache Veraschung Methods (Säuregemisch-Veraschung). Z. Physiol. Chem., Bd. 37, S 115.

PORTER-LEVIN, T. 1933 Calcium and phosphorus metabolism of normal preschool children II. Successive balance studies showing the range of variation in calcium and phosphorus storage. J. Am. Diet. Assoc., vol. 9, p. 22.

PYLE, S. I., AND C. E. HUFF (WITH R. DAVIS) 1936 The use of 3-day periods in human metabolism studies. Calcium and phosphorus. J. Nutrition, vol. 11, p. 495.

SHERMAN, H. C. 1937 Chemistry of Food and Nutrition 5th ed. The Macmillan Co., New York, p. 640.

SHOHL, A. T., AND A. SATO 1923 Acid-base metabolism. II. Mineral metabolism. J. Biol. Chem., vol. 58, p. 257.

A LONG-TIME STUDY OF NITROGEN, CALCIUM AND PHOSPHORUS METABOLISM ON A MEDIUM-PROTEIN DIET[1]

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INTRODUCTION

Considerable fluctuation in nitrogen, calcium and phosphorus retentions of three young women on a constant low-protein diet supplying 0.42 gm. protein per kilogram over a 45-day period was reported in a previous paper (Kunerth and Pittman, '39). Because smaller fluctuations in nitrogen retention might be expected on a higher protein level, a diet three times as high in protein divided into fifteen 3-day experimental periods, as in the other study, was planned to determine the extent and nature of these differences.

The three women on the previous experiment and an additional subject ate a diet similar to that of the low-protein study. Subjects A and I received 40, and M and B 45 Calories per kilogram. All subjects were to receive twice their requirement of protein as recommended by Sherman ('37) and again 85% was to be furnished by meat. The calcium and phosphorus levels were not changed appreciably.

The higher protein allowance permitted a somewhat more liberal diet (table 1). Graham crackers and butter were substituted for the agar cracker and butterfat previously used and apples were added. The procedure used for preparing and serving the food, collection of samples and methods of analysis were the same as reported in the previous paper.

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[1] Contribution no. 82.

TABLE 1

Daily food intakes on a medium-protein diet[1]

SUBJECT	COMPOSITION OF DIET	BEEF ROUND	BEVERAGE[2]	BUTTER	CABBAGE SALAD[3]	GRAHAM CRACKERS	APPLE	AGAR	$CaH_4(PO_4)_2 \cdot H_2O$	$Ca(C_3H_5O_3)_2 \cdot 5H_2O$	$FeC_6H_5O_7 \cdot 3H_2O$	NaCl	TOTAL
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
M	Weight	243.0	593.0	78.0	113.1	43.2	119.3	1.0	0.898	1.092	0.0316	2.000	9.85
	Nitrogen	8.63	0.33	0.12	0.16	0.57	0.04						0.409
	Calcium	0.016	0.045	0.008	0.030	0.014	0.011		0.143	0.142			0.879
	Phosphorus	0.485	0.068	0.012	0.023	0.056	0.014		0.221				3.63
	Fiber				0.79	0.65	1.19	1.00					51.40
	Ml. excess N. acid	33.79				3.37			14.24				37.41
	Ml. excess N. base		22.07		3.45		4.49			7.07	0.33		
B	Weight	254.0	611.0	78.0	113.1	47.5	119.3	1.0	0.955	1.177	0.0294	2.000	10.30
	Nitrogen	9.02	0.33	0.12	0.16	0.63	0.04						0.431
	Calcium	0.017	0.045	0.008	0.030	0.015	0.011		0.152	0.153			0.921
	Phosphorus	0.507	0.068	0.012	0.023	0.062	0.014		0.235				3.69
	Fiber				0.79	0.71	1.19	1.00					54.18
	Ml. excess N. acid	35.33				3.70			15.15				37.94
	Ml. excess N. base		22.07		3.45		4.49			7.62	0.31		
A	Weight	274.0	608.0	104.0	79.4	50.1	119.3	1.0	1.048	1.301	0.0268	2.000	11.08
	Nitrogen	9.72	0.33	0.17	0.16	0.66	0.04						0.467
	Calcium	0.018	0.045	0.010	0.030	0.016	0.011		0.167	0.170			0.986
	Phosphorus	0.546	0.068	0.012	0.023	0.065	0.014		0.258				3.73
	Fiber				0.79	0.75	1.19	1.00					58.63
	Ml. excess N. acid	38.10				3.91			16.62				38.71
	Ml. excess N. base		22.07		3.45		4.49			8.42	0.28		
I	Weight	305.0	596.0	78.0	124.1	64.8	119.3	1.0	1.211	1.561	0.0225	2.000	12.44
	Nitrogen	10.93	0.33	0.12	0.16	0.86	0.04						0.531
	Calcium	0.020	0.045	0.008	0.030	0.021	0.011		0.193	0.203			1.107
	Phosphorus	0.608	0.068	0.012	0.023	0.084	0.014		0.298				3.95
	Fiber				0.79	0.97	1.19	1.00					66.66
	Ml. excess N. acid	42.39				5.06			19.21				40.35
	Ml. excess N. base		22.07		3.45		4.49			10.11	0.23		

[1] Based on analysis of foods by periods.

[2] 150 gm. strained orange juice, 350 gm. commercial grape juice, and 50 gm. lactose for all subjects. Subject M had 43 gm. sucrose; subject B, 61 gm.; subject A, 58 gm.; and subject I, 46 gm.

[3] All subjects had 79.4 gm. shredded cabbage; subjects M, B and I had 0.7 gm. citric acid; subjects M and B each had 33.0 gm. corn oil; subject I, 44.0 gm.

RESULTS

The subjects remained in good health throughout the experiment and maintained almost constant weights, indicating that their caloric intakes met their energy requirements satisfactorily. Analysis of the foods showed that intakes of nitrogen, calcium, and phosphorus were quite constant throughout the fifteen consecutive 3-day periods.

The averages of basal metabolic tests made weekly throughout the study were —3.0, —9.5, —9.7 and —5.6% for subjects M, B, A and I, respectively (Aub-DuBois standard as modified by Boothby and Sandiford). A comparison of the basal means for the three subjects who had also served in the previous study showed that two were lower for this period and one was higher. Increasing the amount of protein approximately 300% or from 0.42 gm. per kilogram to 1.23 gm. per kilogram did not change the basal metabolic rate consistently nor to any marked degree. This agrees with the statement of Forbes et al. ('35) that increased protein to improve the nutritive value in equicaloric diets of rats "made no change in the basal heat production per unit of computed surface area."

Nitrogen findings. Analysis of the food showed that the diet supplied 194% of the protein requirement or 1.2 gm. per kilogram so that, for an adult, it may be regarded as a medium-protein diet. The meat furnished 87.7% of the nitrogen. The average daily nitrogen intakes for the four subjects, M, B, A and I were 9.85, 10.30, 11.08 and 12.44 gm. (table 2) or 197.4, 198.5, 196.1 and 194.7 mg. per kilogram, respectively, with standard deviations of 0.83, 0.77, 0.72 and 0.66 mg. The maximum variation in nitrogen intakes during the fifteen periods was 3.2 mg. per kilogram for subject M and the minimum was 2.1 for subject A.

The dry weight of the feces for the four subjects averaged 268.5 mg. per kilogram per day 37.3 mg. less than on the low-protein diet. The mean daily per capita fiber intakes of 3.9 gm. for the low and 3.8 gm. for the medium-protein study do not explain this difference. Two subjects actually increased their fiber intake slightly over the earlier study while one subject (A) consumed 22.9% less. All subjects showed decrease in bulk of feces ranging from 6.6% for subject M to 14.0% for subject A.

Fecal nitrogen averaged 56.5 mg. per gram dry feces ranging from 41.8 to 64.3 mg. per gram for the different subjects.

TABLE 2

Average daily nitrogen, calcium and phosphorus balances on a medium-protein diet

SUBJECT	WEIGHT OF SUBJECT	AVERAGE DAILY INTAKE		AVERAGE DAILY OUTPUT			BALANCE	
		Meat	Total	Urine	Feces	Total	Total	Per kilogram
				Nitrogen				
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*
M	49.9	8.63	9.85	7.50	0.84	8.34	+1.51	+30.3
B	51.9	9.02	10.30	7.94	0.89	8.83	+1.47	+28.3
A	56.5	9.72	11.08	8.70	0.82	9.52	+1.56	+27.6
I	63.9	10.93	12.44	9.85	0.77	10.62	+1.82	+28.5
Mean	55.6	9.58	10.92	8.50	0.83	9.33	+1.59	+28.8
				Calcium				
M		0.016	0.409	0.116	0.318	0.434	−0.025	− 0.5
B		0.017	0.431	0.164	0.307	0.471	−0.040	− 0.8
A		0.018	0.467	0.128	0.378	0.506	−0.039	− 0.7
I		0.020	0.531	0.100	0.489	0.589	−0.058	− 0.9
Mean		0.018	0.460	0.127	0.373	0.500	−0.040	− 0.7
				Phosphorus				
M		0.485	0.879	0.629	0.252	0.881	−0.002	± 0.0
B		0.507	0.921	0.656	0.267	0.923	−0.002	± 0.0
A		0.546	0.986	0.604	0.253	0.857	+0.129	+ 2.3
I		0.608	1.107	0.734	0.343	1.077	+0.030	+ 0.5
Mean		0.537	0.973	0.656	0.279	0.935	+0.039	+ 0.7

This approximated the mean of 54.9 mg. of the low-protein diet, but the latter was less constant. This slightly higher excretion of fecal nitrogen per gram of dry feces on the medium-protein diet was balanced by the slightly smaller amount of feces, so the mean total nitrogen excreted in the feces was almost the same for the two levels of protein, being 0.86 gm. per capita daily for the low and 0.83 gm. for the medium-protein diet. This agrees with the findings of Hawks et al.

('38) with children that a fairly constant amount of nitrogen was excreted in the feces and that it apparently had little relation to variations in intake of nitrogen.

The percentage of nitrogen intake excreted in the urine was increased from 68.3% on the low-protein to 77.8% at the medium level. That excreted in the feces was correspondingly lower amounting to 21.4 and 7.6%, respectively. The mean total nitrogen excretion representing 90.0% of the intake on the low-protein fell to 85.4% with the medium-protein intake.

Hawks' study of five children indicated that on constant diets containing 3 gm. protein per kilogram, the same proportion of the nitrogen intake was excreted. This was apparently true of the four subjects of this study, the percentage of the total nitrogen excreted being 84.7, 85.7, 85.9 and 85.4%. It was less true of the three subjects on the low-protein diet, the percentage of intake excreted ranging from 86.0 to 95.2%. This suggests that the total nitrogen excretion becomes more constant as the protein is increased in the diet.

The apparent coefficient of digestibility was increased on the medium-protein diet to a mean of 92.4% compared with 78.6% on the low-protein, a difference of 13.8%. This agrees with Murlin and Mattill ('38) that lower values in a low-protein diet may be attributed to the low level of protein in the diet, which is not favorable to a high coefficient of digestibility. Forbes et al. ('35) found increased efficiency of digestion of protein in rats by increasing the protein content of equicaloric diets. Hawks et al. ('38) reported an increase of as much as 2.6% in coefficients of digestibility for children when changed from a medium- to a high-protein diet. As protein standards for children are higher than those of adults, it must be remembered that her medium level of 3 gm. protein per kilogram was higher than that of this study of adults of 1.2 gm. per kilogram.

The mean coefficient of digestibility ranged from 91.4 to 93.8% for the various subjects although B dropped to a low of 88.3% and I reached a high of 95.8% in one period. Subject M showed the least difference in coefficients of digestibility during the fifteen periods (4.7%) and subject I the most (6.9%). The mean nitrogen absorption was 181.5 mg. per kilogram compared with 61.0 mg. in the low-protein study.

The medium-protein diet increased the apparent coefficient of digestibility of the nitrogen by decreasing the proportion of intake excreted in the feces and increasing that in the urine. The retention was higher and the percentage of intake retained increased slightly.

Hawks et al. ('38) found change from a medium- to a high-protein diet did not increase the percentage of intake stored by children. With these adult women subjects, nitrogen retention was higher on the medium-protein diet increasing from 7.8 mg. per kilogram or 10.1% of the intake on the low-protein to 28.7 mg. per kilogram or 14.6% of the intake on the medium-protein diet. The mean nitrogen retention for all subjects was +1.59 gm. (table 2) daily, ranging from an average of +1.47 gm. for subject B to +1.82 gm. for subject A. In all of the sixty periods for the four subjects (table 3) there was but one negative nitrogen balance (0.8 mg. per kilogram per day for subject M) which may be considered equilibrium. The highest daily retention was +52.1 mg. per kilogram for subject I for period 6 although all subjects at numerous times had retentions exceeding 2 gm. daily. These unexpectedly high retentions by these young adult women continued throughout the study though the average for the total amount retained was 3.35 gm. higher in the first half of the experiment, perhaps indicating a slight tendency to decreased storage as the diet was continued. Subject I, 31 years of age, retained an average of 28.5 mg. per kilogram daily which, when compared with the mean retention of 28.7 mg. per kilogram for the entire group, showed no tendency to decrease the storage with greater maturity.

Calcium findings. The average daily calcium intakes were 0.409, 0.431, 0.467 and 0.531 gm. for M, B, A and I, respectively, or, expressed in milligrams per kilogram, 8.20, 8.30, 8.27 and 8.31 with standard deviations of 0.15, 0.14, 0.14 and 0.12 mg.

TABLE 3

Average daily nitrogen, calcium and phosphorus retentions per kilogram on a medium-protein diet

PERIOD	SUBJECT M			SUBJECT B			SUBJECT A			SUBJECT I		
	N	Ca	P	N	Ca	P	N	Ca	P	N	Ca	P
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	+33.3	—1.0	—0.3	+30.1	—1.9	±0.0	+20.5	—2.0	—0.7	+16.7	— 6.0	—3.5
2	+37.5	—1.4	+0.5	+36.6	—0.7	—0.1	+29.9	+0.8	+1.3	+26.6	— 3.9	—0.9
3	+13.9	—1.4	—1.5	+19.4	—2.0	—0.1	+40.4	—0.2	+1.0	+29.4	— 1.0	+1.0
4	+20.5	—0.4	—2.0	+22.9	—0.4	—0.1	+29.9	+0.8	—0.2	+30.2	— 1.8	±0.0
5	+30.6	+0.1	+0.1	+20.3	—1.3	—1.6	+23.9	—0.7	+2.6	+34.6	+ 0.3	+1.0
6	+36.0	+0.6	—0.4	+28.6	—0.4	—0.7	+22.5	—0.7	+3.3	+52.1	+ 1.1	+1.9
7	+47.3	+1.2	+2.1	+36.5	+0.4	+0.8	+39.8	—2.3	+2.9	+29.0	+ 0.3	+0.7
8	+29.6	—1.0	—0.3	+31.5	—0.7	—0.1	+32.2	—0.1	+3.0	+25.2	— 1.9	+0.2
9	+31.8	+1.0	+1.7	+32.0	—0.3	—0.9	+16.6	—1.5	—0.6	+24.9	— 0.6	+0.6
10	+39.3	+0.4	+2.8	+34.2	+0.7	+1.2	+19.1	+0.5	+5.0	+12.1	— 0.6	+1.1
11	+17.2	—0.8	+0.4	+36.4	—1.0	+0.4	+29.4	—0.9	+2.2	+39.3	— 2.3	+2.1
12	+37.5	+0.3	+0.8	+41.0	+0.2	+1.9	+37.5	—0.3	+5.1	+19.1	— 2.4	—2.0
13	— 0.8	—1.9	—3.3	+32.5	+1.4	+2.6	+29.7	—1.3	+4.0	+12.4	± 0.0	—0.1
14	+39.1	—1.6	—0.7	+15.7	—2.2	—1.5	+22.1	+1.5	+3.0	+39.6	+ 2.4	+2.1
15	+42.3	—1.4	—0.5	+ 6.1	—3.2	—2.4	+21.2	—3.6	+3.2	+36.8	+ 2.4	+2.7
Mean	+30.3	—0.5	±0.0	+28.3	—0.8	±0.0	+27.6	—0.7	+2.3	+28.5	— 0.9	+0.5
						Per cent of intake						
Mean	15.3	—6.1	0.0	14.3	—9.6	0.0	14.1	—8.5	13.1	14.6	—10.8	2.9

The largest variation in calcium intake during the fifteen periods, 0.52 mg. per kilogram, occurred in the case of subject M. Subject I had the least variation amounting to 0.42 mg. per kilogram.

The average calcium output for all subjects was 0.500 gm. per day or 9.0 mg. per kilogram which resulted in an average retention of —0.7 mg. per kilogram. The average daily fecal calcium for all subjects was 6.7 and the urinary calcium was 2.3 mg. per kilogram but the amounts varied considerably throughout the study. For the fifteen periods subjects M, B, A and I excreted 73, 65, 75 and 83% of the total calcium output in the feces.

Phosphorus findings. Subjects M, B, A and I received daily 0.879, 0.921, 0.986 and 1.107 gm. of phosphorus or 17.6, 17.7, 17.5 and 17.3 mg. per kilogram with standard deviations of 0.17, 0.14, 0.13 and 0.13 mg. A maximum variation of 0.65 mg. per kilogram for subject M and a minimum of 0.46 mg. for subject I occurred in phosphorus intakes.

The subjects excreted 0.881, 0.923, 0.857 and 1.077 gm. per day, indicating that over the 45-day period subjects M and B were in equilibrium and subjects A and I were storing phosphorus, particularly A. The average fecal phosphorus amounted to 28.6% of the intake and ranged from a low of 4.5 mg. per kilogram for subject A to a high of 5.4 mg. per kilogram for subject I. Of the total output, 71% was urinary phosphorus for subjects M, B and A, whereas for I, it was 68%.

In the previous experiment in which three of these subjects had a moderately low protein diet but received 6.8% more calcium and 4.3% more phosphorus, all had average negative calcium balances for the study and two had negative phosphorus balances. In the present study these same subjects had less negative calcium balances (6.4, 12.4 and 9.9%, respectively) and their phosphorus retentions improved in two cases and in the third remained at approximate equilibrium. A comparison of the urinary and fecal calcium outputs of the three subjects serving on both diets showed that the smaller negative balances on the higher protein diet were due to smaller fecal loss, as the urinary calcium was about the same for both diets. To a somewhat less extent, the same is true for phosphorus.

The improved calcium retention can scarcely be explained on the basis of the difference in the excess acid of the two diets as the former had about twice as much excess acid as the higher protein diet. Davis ('35) in comparing retention and path of excretion of nitrogen, calcium and phosphorus of children on acid-and-base forming diets found greatest effect on calcium. However, her results indicate that on a basic diet the proportion of fecal calcium was increased and urinary calcium decreased but retention was not influenced.

The fiber content of the two diets differed little except for subject A, but its effect on the mineral utilization is doubtful. In the low-protein diet subject A had more fiber in the diet than the other subjects and excreted more fecal calcium but less phosphorus than the others. In the present study she had about the same amount of fiber as the other subjects, about a fourth less than she had had on the previous diet, but again she had higher fecal calcium and lower phosphorus than subjects M and B. Ascham ('30–'31) found some evidence that fecal calcium in dogs was increased by increased amounts of agar but discrepancies occurred. Some attention has been given to mineral utilization on protein enriched diets in other studies. Conner and Sherman ('36) found, with increase of protein in the diet from 14.0 to 18.8%, a gain in body calcium in 2- to 3-month-old rats receiving minimum adequate calcium allowances. However, Woodman et al. ('37) in a study of the nutrition of the bacon pig found no differences in mineral retention that might be correlated with an increase from a normal to a high level of protein intake.

A total of six menstrual periods occurred during the study: two each for subjects M and A, and one each for subjects B and I. Retentions were above the average for the study during the menstrual periods in all six cases for nitrogen, in five for calcium, and in four for phosphorus. No particularly unfavorable effects on retentions could be noted at these times.

The higher intake of protein gave somewhat better retentions than were noted on the low-protein diet of the earlier study. No menstrual collections were made. What changes analyses of the menstrua would have made cannot be predicted.

CONCLUSIONS

1. A medium-protein diet did not eradicate the tendency to variation in retention of nitrogen, calcium and phosphorus observed in the low-protein diet of a previous study.
2. A constant diet furnishing 1.2 gm. protein (87.7% from beef) and 40 to 45 Calories per kilogram enabled four young adult women to maintain a positive balance for nitrogen and phosphorus during forty-five consecutive balance days divided into fifteen periods of 3 days each. No subjects were in positive calcium balance, though improved retention over the low-protein study was noted.
3. The medium-protein diet used in this study improved appreciably the utilization of nitrogen, calcium and phosphorus by human subjects compared with the low-protein diet of the earlier investigation.

With this increase in protein the level used for calcium was inadequate to maintain these subjects under the conditions of this study in equilibrium during a 45-day period.

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LITERATURE CITED

Ascham, L. 1930–1931 The influence of bulk in the diet upon fecal calcium and phosphorus. J. Nutrition, vol. 3, p. 411.

Conner, R. T., and H. C. Sherman 1936 Some aspects of protein intake in relation to growth and rate of calcification. J. Biol. Chem., vol. 115, p. 695.

Davis, N. J. 1935 Calcium, phosphorus and nitrogen retention of children. Effects of acid-forming and base-forming diets. Am. J. Dis. Child., vol. 49, p. 611.

Forbes, E. B., R. W. Swift, A. Block and O. J. Kahlenberg 1935 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. III. The effects of the plane of protein intake. J. Nutrition, vol. 10, p. 461.

Hawks, J. E., M. M. Bray and M. Dye 1938 The influence of diet on the nitrogen balances of pre-school children. J. Nutrition, vol. 15, p. 125.

Kunerth, B. L., and M. S. Pittman 1939 A long-time study of nitrogen, calcium and phosphorus metabolism on a low-protein diet. J. Nutrition, vol. 17, p. 161.

Murlin, J. R., and H. A. Mattill 1938 Digestibility and nutritional value of cereal proteins in the human subject. J. Nutrition, vol. 16, p. 15.

Sherman, H. C. 1937 Chemistry of Food and Nutrition. 5th ed. The Macmillan Co., New York, p. 640.

Woodman, H. E., R. E. Evans and W. G. Turpitt 1937 The nutrition of the bacon pig. II. The influence of high-protein intake on protein and mineral metabolism. J. Agr. Sci., vol. 27, p. 569.

A STUDY OF BREED AND SEASONAL VARIATIONS IN THE ASCORBIC ACID CONTENT OF CERTIFIED MILK FROM GUERNSEYS AND HOLSTEINS

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ONE FIGURE

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The ascorbic acid content of cow's milk has been studied by many investigators. Barnes and Hume ('19) found a seasonal variation in the antiscorbutic properties of cow's milk. In 1920 Dutcher, Eckles, Dahle, Mead and Schaefer reported that the vitamin content of cow's milk is dependent upon the vitamin content of the ration ingested by the cow. Hart, Steenbock and Ellis ('20) concluded that summer pasture milk is much richer in the antiscorbutic vitamin than dry feed milk or winter milk, produced with a ration which included corn silage or sugar mangels. Hess, Unger and Supplee ('20) assayed dried milks and found that the milk from cows on pasture serves as a fair source of vitamin C whereas the milk from cows receiving a feed low in vitamin C possessed practically no antiscorbutic properties.

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Olson and Copeland ('24) stated that the vitamin C in milk has its origin in the feed of the cow, and that good silage is important in order to maintain an adequate supply of this vitamin in the milk.

MacLeod ('27) reported that "the greater antiscorbutic value of the milk of the pasture-fed cow is doubtless due to the antiscorbutic vitamin content of the fresh grasses which the animal receives, since the animal body probably does not synthesize this vitamin and is therefore dependent on its food to provide an adequate amount of it in its milk."

Rasmussen, Guerrant, Shaw, Welch and Bechdel ('36) stated that the ascorbic acid content of milk is apparently dependent solely upon the ascorbic acid content of the diet after the first 2 months of lactation. Hannisdahl ('37) reported that the ascorbic acid content of the milk from stall-fed cows was lower than that from pasture-fed cows.

On the other hand, Hughes, Fitch, Cave and Riddell ('27) concluded from their experiments "that the vitamin C content of a cow's ration has little if any influence on the vitamin C content of its milk." These investigators also stated the dairy cow can synthesize vitamin C to some extent.

In 1936 Riddell, Whitnah, Hughes and Lienhardt, in a study concerning the influence of rations on the vitamin C content of milk found that the rations studied had no significant influence on the vitamin C content of cow's milk. Whitnah and Riddell ('37) fed three cows approximately 65 kg. per day of green rye which contained 0.86 mg. of vitamin C per gram and found that "15 days after the initial heavy rye feeding there was no significant increase in the vitamin C concentration of the milk from any of the three cows." Reedman ('37) reported that dairy cattle apparently have a mechanism for synthesizing ascorbic acid and therefore are not susceptible to vitamin C deficiency. West and Wenger ('38) found no apparent difference in the ascorbic acid content of the cow's milk after the addition of orange meal to the feed.

Unfortunately, the majority of the conclusions cited above are based upon the results obtained from studies of only a few days to a few months in duration. Furthermore in some cases the data have been gathered from experiments which included only two or three cows. In view of the short experimental periods, the small number of cows employed in most of the experiments and the lack of agreement in the published results, it seemed desirable to conduct a study of the vitamin C content of certified milk, produced under controlled conditions, by a group of twenty-five to thirty cows during a complete year.

Twenty-eight typical Holstein and Guernsey cows were selected for this study from a herd of 200 cows maintained under carefully controlled conditions for the production of certified milk. The experimental cows were housed with the remainder of the herd in modern dairy barns throughout the period of the experiment. They were continuously stall-fed and at no time had access to pasture. To guard against disease the cows were under the direct supervision of an experienced veterinarian, the attendants were under constant medical inspection, and before a cow was added to the herd she was subjected to a 60-day quarantine in an isolation barn in order to eliminate any animal affected with mastitis, infectious abortion or other contagious bovine diseases.

From time to time it was necessary to remove cows from the study on account of diminished milk flow. These were replaced by representative cows selected from the certified milk herd. When the cows that had been removed from the study again freshened they were returned to the experiment. Hence at all times during this investigation the experimental animals consisted of cows which represented practically all stages of lactation. As a result of replacing cows during non-lactating periods, fifty-five different cows were used in this study. Accordingly the results obtained should be fairly applicable to certified milk produced by the best managed commercial dairies.

Obviously data concerning the age, size, physical condition and number of pregnancies of the cows used are of interest for evaluating the results of this study. The age of the Guernsey cows varied from 4 to 11 years and that of the

Holstein cows from 5 to 9 years. The weight of the Guernsey cows varied from 840 pounds to 1170 pounds whereas the Holstein cows varied from 885 pounds to 1300 pounds. The average weight of the Holstein cows (1088 pounds) was materially greater than that of the Guernsey cows (972 pounds). All of the cows, except three or four Holsteins, were in very satisfactory physical condition. Seven cows were in their second lactation period and three were in their sixth lactation period but the great majority were in their third or fourth lactation periods.

All cows received a daily ration compounded from various combinations of the following ingredients: first and second grades of alfalfa hay, first and second grades of mixed hay, beet pulp, dairy ration, dairy ration with 6.7% irradiated yeast, home mixed fitting ration with 6.7% irradiated yeast and a commercial fitting ration with irradiated yeast. The composition of the dairy and fitting ration is reported in table 1. Samples of these products were assayed for their ascorbic acid content by Tillman's method as modified by Bessey and King ('33). Five gram samples were ground with 30 gm. of acid-washed sand; 25 to 35 cc. of an acid mixture containing 4% metaphosphoric and 8% acetic acid was added as suggested by Musulin and King ('36). The mass was centrifuged, the supernatant liquid was decanted and the residue repeatedly extracted until 50 cc. of extract were obtained. Five cubic centimeters of the extract, diluted to 25 cc. were titrated with 2,6-dichlorophenolindophenol as described by Tripp, Satterfield and Holmes ('37). The results of these assays are reported in table 2.

These values are less than those of Reedman ('37) who reported that pasture grass contained 20.5 to 136.0 mg. total ascorbic acid per 100 gm. He also mentioned that hay may contain as much as 30 mg. ascorbic acid per 100 gm., whereas in the case of ensilage 12% to 50% of the original ascorbic acid may be preserved for a period of 6 months depending upon the method of preservation. Reedman estimates that a dairy cow on winter ration receives approximately 9 gm. of ascorbic acid daily in the ration.

TABLE 1

Composition of dairy and fitting rations

Dairy ration	%
Wheat bran	25.0
Oats (crimped)	17.5
Yellow hominy feed	10.0
Distillers' corn grains dried	10.0
Corn gluten feed	10.0
Cottonseed meal, 41% (hydrolic process 5% fat)	10.0
Yellow cornmeal	5.0
Soy bean oil meal (expeller process)	5.0
Cane molasses	5.0
Iodized salt	1.0
Steamed bone meal	1.0
Calcium carbonate	0.5
Home mixed fitting ration	
Oats (crimped)	30.0
Wheat bran	25.0
Yellow hominy feed	15.0
Yellow cornmeal	10.0
Soy bean oil meal (expeller process)	10.0
Cane molasses	7.0
Steamed bone meal	1.0
Calcium carbonate	1.0
Iodized salt	1.0
Commercial fitting ration	
Cornmeal	Unknown
Corn gluten meal	Unknown
Crushed oats	Unknown
Soy bean oil meal	Unknown
Wheat middlings	Unknown
Wheat bran	Unknown
Alfalfa meal	Unknown
Dried beet pulp	Unknown
Molasses	Unknown
Calcium carbonate	Unknown
Iodized salt	Unknown
Carotene	Unknown

EXPERIMENTAL

Samples of morning milk were taken from each of the twenty-eight cows at bi-monthly intervals during the entire experimental year. The representative samples were placed in 1 ounce flint glass bottles which were immediately placed on ice and protected from light. The milk samples were tested by the indophenol titration method employing 2,6-dichlorophenolindophenol. The vitamin C was determined by the method of King ('37). Five cubic centimeters of milk were added to a 2.5 cc. mixture of 8% acetic acid and 4% metaphosphoric acid in a 50 cc. Erlenmeyer flask. The dye solution was added from a microburette drop by drop until a faint pink end point was reached which remained for 30 seconds. The strength of the dye solution was equivalent to 0.08 to 0.10 mg. of vitamin C per cubic centimeter. This solution was prepared and standardized in a manner similar to that described by Tripp, Satterfield and Holmes ('37).

TABLE 2

The ascorbic acid content of the components of the experimental ration

	Ascorbic acid per 100 gm. of feed mg.
Good farm hay (one-half alfalfa)	1.334
Second grade farm hay (all grass)	0.907
Grass ensilage	1.548
First grade baled alfalfa	1.883
Second grade baled alfalfa	1.380
Beet pulp	0.669
Dairy ration	0.562
Dairy ration with irradiated yeast	0.754
Fitting ration and irradiated yeast	0.592
Commercial fitting ration with irradiated yeast	1.622

RESULTS

The results of the bimonthly assays of certified milk are reported in table 3 which shows the number of cows producing milk, the minimum, maximum and average ascorbic acid content of the Guernsey and Holstein milks at each assay period. The number of Guernsey cows under observation varied from 10 to 15 but usually there were thirteen or fourteen, and the number of Holsteins varied from eleven to sixteen with an average of fourteen. All the values for ascorbic acid refer to the milligrams per 1000 cc. of milk. The minimum ascorbic acid content of Guernsey milk varied from 11.07 mg. on December 9th to 19.31 mg. on August 11th, and the maximum varied from 21.66 mg. on May 6th to 31.37 mg. on October 14th with averages varying from 17.76 mg. on May 6th to 23.37 mg. on February 17th. The minimum ascorbic acid content of Holstein milk varied from 9.84 mg. on April 21st to 16.73 mg. on October 28th; the maximum varied

TABLE 3

Ascorbic acid content of certified cow's milk (milligrams per 1000 cc. milk)

GUERNSEYS					HOLSTEINS				
Date	Number of cows	Minimum	Maximum	Average	Date	Number of cows	Minimum	Maximum	Average
Aug. 19	11	15.81	24.63	18.93	Aug. 19	11	14.34	21.32	17.55
Sept. 16	14	17.24	29.81	20.26	Sept. 16	14	15.45	21.55	18.05
Sept. 30	14	12.32	25.80	18.63	Sept. 30	14	15.02	22.33	18.95
Oct. 14	13	19.21	31.37	21.75	Oct. 14	12	14.51	23.13	19.46
Oct. 28	14	16.74	27.50	21.29	Oct. 28	13	16.73	25.27	20.44
Nov. 10	14	18.69	25.70	21.96	Nov. 10	14	16.35	24.76	20.02
Nov. 24	14	16.95	27.03	21.41	Nov. 24	14	15.95	21.82	19.48
Dec. 9	15	11.07	27.68	20.86	Dec. 9	14	15.30	23.44	19.72
Dec. 23	14	16.24	22.74	19.38	Dec. 23	14	12.35	22.42	17.18
Jan. 6	14	18.52	23.72	21.26	Jan. 6	14	13.97	20.47	18.24
Jan. 20	14	18.98	27.00	22.59	Jan. 20	14	13.50	22.99	19.62
Feb. 3	14	18.63	26.00	21.92	Feb. 3	14	14.05	23.54	19.53
Feb. 17	14	19.10	28.23	23.37	Feb. 17	15	15.78	24.91	20.23
Mar. 3	14	18.30	25.16	21.05	Mar. 3	15	13.72	20.97	18.22
Mar. 23	14	14.95	23.77	20.31	Mar. 23	15	11.50	19.94	16.89
Apr. 7	14	15.22	23.02	19.22	Apr. 7	14	11.88	19.68	16.82
Apr. 21	14	13.78	22.30	19.35	Apr. 21	14	9.84	19.35	15.72
May 6	14	13.97	21.66	17.76	May 6	15	11.52	18.15	16.04
May 19	13	17.02	24.03	20.36	May 19	16	11.68	21.36	17.82
June 2	13	17.61	22.09	19.47	June 2	15	10.70	19.33	16.89
June 23	13	17.83	23.65	20.12	June 23	15	10.97	19.88	16.55
July 8	13	15.85	22.93	20.30	July 8	15	14.84	21.60	17.70
July 21	13	15.50	22.14	18.45	July 21	14	14.62	19.50	16.99
Aug. 11	10	19.31	25.48	22.84	Aug. 11	12	14.05	21.08	17.89
Average	13	16.62	25.14	20.54	Average	14	13.69	21.62	18.17

from 18.15 mg. on May 6th to 25.27 mg. on October 28th with averages varying from 15.72 mg. on April 21st to 20.44 mg. on October 28th.

The average ascorbic acid content of the 324 samples of Guernsey milk assayed during the year's experiment was 20.54 mg. and that of the 337 samples of Holstein milk was 18.17 mg. These data are in agreement with those recently published by West and Wenger ('38) who found that Guernsey milk was superior to that from the Holstein cow as a source of vitamin C. Rasmussen ('36) also found that milk from Holstein cows was inferior to that produced by Brown Swiss, Ayrshire, Guernsey and Jersey cows as sources of vitamin C.

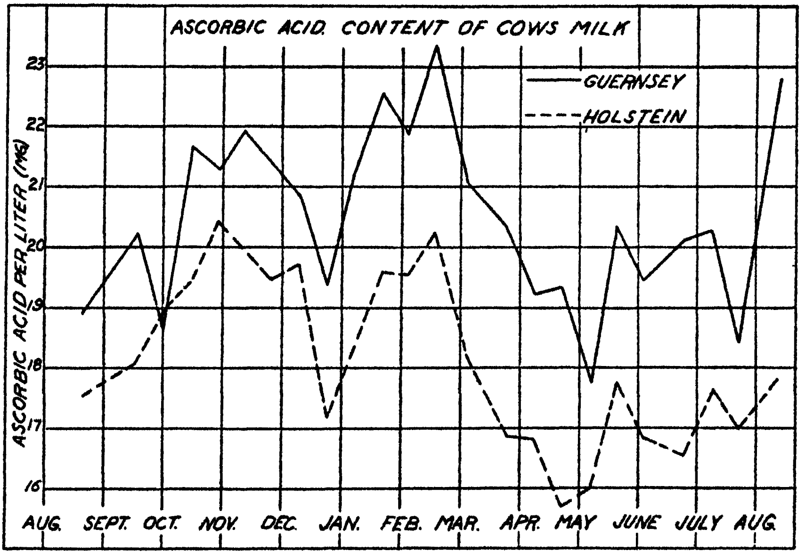

Fig. 1 The ascorbic acid content of certified milk as influenced by breeds and seasons.

The seasonal variations in certified milk produced by stall-fed cows maintained under conditions which were uniform for the entire year are shown by the graph on figure 1. An examination of figure 1 shows that the amount of ascorbic acid found in milk of both the Guernsey and Holstein cows tended to be greater during October, January and February than during the other periods of the year, although there was a pronounced increase in vitamin C content of the Guernsey milk on the last day of the experiment. From the middle of December until the middle of February there appeared to be a steady and consistent increase in the ascorbic acid content of the milk of both breeds. From the middle of February until early May there was a gradual and persistent drop in the amount of ascorbic acid in both the Guernsey and Holstein milks. However, the composition of the ration was not altered previous to or during this period. The amount of vitamin C in the milk of both breeds was at its lowest point in early May. Seasonal variations in cows' milk have been observed by a number of investigators. Rohmer, Bezssonoff, Sanders and E. Stoerr ('34), in a study involving cow's milk from three different sources, found that the vitamin C content was high in November, dropped sharply in December and rose in March and April. Sharp ('36) reported that the ascorbic acid content of fresh milk is relatively constant throughout the year. Whitnah and Riddell ('37) found that the ascorbic acid content for a herd of cows increased from October to December, decreased from December to February and increased again from February to March.

On the other hand some investigators have obtained contrary results. Kon and Watson ('37) stated that the level of vitamin C in mixed milk is very constant and is independent of the season of the year. MacLeod ('27) concluded "that the milk of stall-fed cows which are given a well balanced, uniform diet throughout the year will not show seasonal variations in antiscorbutic value."

It is interesting to note in connection with the seasonal variation in the ascorbic acid content of cow's milk observed in this study that Sinkko ('37) also observed a seasonal variation in the ascorbic acid content of human milk collected from ten women at intervals throughout a year. In the present study the certified milk collected in February and early March contained the largest amount of ascorbic acid, whereas Sinkko found the human milk to have the highest vitamin C content in August and September. Incidentally Sinkko observed that the incidence of still-births and infant mortality were lowest at the season of the year when the mothers were less likely to be vitamin C deficient.

The amounts of ascorbic acid in Guernsey and Holstein milks (20.54 and 18.17 mg. respectively) reported in this paper are greater than those observed by West and Wenger ('38). These workers concluded that raw certified milk can contribute materially to the vitamin C content of the human dietary, particularly since they observed only a 17% loss in the ascorbic acid content 34 hours after milking.

SUMMARY

Six hundred and sixty-one samples of certified milk collected at bi-monthly intervals from fifty-five Guernsey and Holstein cows over a 52-week period were assayed for their ascorbic acid content by the dichlorophenolindophenol titration method.

The ascorbic acid content of both Guernsey and Holstein milk showed seasonal variation. The February milk contained large amounts of ascorbic acid, 23.37 mg. for the Guernsey and 20.23 mg. per liter for the Holstein milk. A second peak occurred in early November when the ascorbic acid content of the Guernsey milk was 21.96 mg. and late October when the ascorbic acid content of the Holstein milk was 20.44 mg.

The average ascorbic acid content of the Guernsey milk varied from 17.76 mg. to 23.37 mg. and for the Holstein milk from 15.72 mg. to 20.44 mg. per liter. The ascorbic acid content was consistently higher for the Guernsey than for the Holstein milk. The total yearly average for the Guernsey milk was 20.54 mg. of ascorbic acid whereas that of the Holstein milk was 18.17 mg. per liter.

The data obtained in this investigation indicate that the variation in the ascorbic acid content of raw certified milk produced under commercial conditions is independent of

ration and period of lactation. However, it is evident from the above results that the generous consumption of raw certified milk can contribute materially to the vitamin C content of the human dietary.

ACKNOWLEDGMENT

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LITERATURE CITED

BARNES, R. E., AND E. M. HUME 1919 A comparison between the antiscorbutic properties of fresh, heated and dried cow milk. Lancet, vol. II, p. 323.

BESSEY, O. A., AND C. G. KING 1933 The distribution of vitamin C in plant and animal tissues, and its determination. J. Biol. Chem., vol. 103, p. 687.

DUTCHER, R. A., C. H. ECKLES, C. D. DAHLE, S. W. MEAD AND O. G. SCHAEFFER 1920 The influence of diet of the cow upon the nutritive and antiscorbutic properties of cow's milk. J. Biol. Chem., vol. 45, p. 119.

HANNISDAHL, B. 1937 Cow's milk as a source of vitamin C. Tidsskr. Norske Laegeforening, vol. 57, p. 1068 (Nutr. Abs. and Revs., vol. 8, p. 89, 1938).

HART, E. B., H. STEENBOCK AND N. R. ELLIS 1920 Influence of diet on the antiscorbutic potency of milk. J. Biol. Chem., vol. 42, p. 383.

HESS, A. F., L. J. UNGER AND G. C. SUPPLEE 1920 Relation of fodder to the antiscorbutic potency and salt content of milk. J. Biol. Chem., vol. 45, p. 229.

HUGHES, J. S., J. B. FITCH, H. W. CAVE AND W. H. RIDDELL 1927 Relation between the vitamin C content of a cow's ration and the vitamin C content of its milk. J. Biol. Chem., vol. 71, p. 309.

KING, C. G. 1937 Personal communication.

KON, S. K., AND M. B. WATSON 1937 The vitamin C content of cow's milk. Biochem. J., vol. 31, p. 223.

MACLEOD, F. L. 1927 Antiscorbutic vitamin content of milk of stall-fed cows through a year. Probability that ensilage is an important source of this vitamin. J. Am. Med. Assoc., vol. 88, p. 1947.

MUSULIN, R. R., AND C. G. KING 1936 Metaphosphoric acid in the extraction and titration of vitamin C. J. Biol. Chem., vol. 116, p. 409.

OLSON, T. M., AND L. COPELAND 1924 The influence of pasteurization and diet of the cow on the antiscorbutic potency of the milk. J. Dairy Sci., vol. 7, p. 370.

RASMUSSEN, R., N. B. GUERRANT, A. O. SHAW, R. C. WELCH AND S. I. BECHDEL 1936 The effects of breed characteristics and stages of lactation on the vitamin C (ascorbic acid) content of cow's milk. J. Nutrition, vol. 11, p. 425.

REEDMAN, E. J. 1937 The ascorbic acid content of milk. Canadian Pub. Health J., vol. 28, p. 339.

ROHMER, P., N. BEZSSONOFF, U. SANDERS AND E. STOERR 1934 Variation saisonniere de la teneur du lait en vitamin C. Extrait du volume des discussions des rapports et communications du VIII^e Congres de l'Association Francaise de Pediatric, Paris, 1934.

RIDDELL, W. H., C. H. WHITNAH, J. S. HUGHES AND H. F. LIENHARDT 1936 Influence of the ration on the vitamin C content of milk. J. Nutrition, vol. 11, p. 47.

SHARP, P. F. 1936 Vitamin C in pasteurized milk. Science, vol. 84, p. 461.

SINKKO, E. I. 1937 Relation between infantile mortality, stillbirth and variations in the vitamin C content of the mother's milk at various seasons of the year in Finland. Acta paediat., vol. 21, p. 407. (Abs. in Nutri. Abs. and Revs., 1938, vol. 8, p. 201.)

TRIPP, F., G. H. SATTERFIELD AND A. D. HOLMES 1937 Varietal differences in the vitamin C (ascorbic acid) content of tomatoes. J. Home Economics, vol. 29, p. 258.

WEST, F. V., AND J. C. WENGER 1938 The vitamin C content of certified milk at the time of consumption. Am. J. Digest. Dis., vol. 5, p. 251.

WHITNAH, C. H., AND W. H. RIDDELL 1937 Variations in the vitamin C content of milk. J. Dairy Sci., vol. 20, p. 9.

THE CALCIUM REQUIREMENTS OF FIVE PRE-SCHOOL GIRLS[1]

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ONE FIGURE

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In 1922 Sherman and Hawley reported that a daily intake of 1 gm. of calcium was necessary to induce optimum storage of this element in children from 3 to 13 years of age. Since that time data on calcium retentions at various levels of intake have been reported for numerous children ranging in age from 3 to 6 years inclusive. Taken as a whole, the observed data afford some evidence that the retentions of calcium on daily intakes of 1 gm. or more of calcium were higher than on intakes of less than 1 gm. However, for the seventy children for whom individual metabolic data are given in the literature (Sawyer, Bauman and Stevens, '18; Willard and Blunt, '27; Wang, Kaucher and Frank, '28; Burton, '29; Wang, Kern and Kaucher, '30; Porter-Levin, '32–'33 and '33–'34; Hunscher et al., '33; Petrunkina, '34; Daniets et al., '34, '35; Hunscher, Hummel and Macy, '36–'37; Bonner et al., '38), great inter- and intra-individual variations in the retentions of calcium were observed.

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[1] Portions of these data were presented before the American Institute of Nutrition, Memphis, Tennessee, April, 1937, and before the American Home Economics Association, Kansas City, Missouri, June, 1937.

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From such inconsistent data it would be hazardous to draw conclusions concerning the minimum quantity of calcium required to insure maximum retention or to predict the quantity of calcium which might be retained on a given level of intake.

The nutritional status of the individual, in regard to the saturation of the bones with calcium, has been suggested as one of the reasons for the variability in retentions among children. Hummel et al., ('37) have reviewed the published evidence for this suggestion and have accepted it as the explanation for the great differences in calcium retentions which they observed in pregnant women. Proof of the contention that the previous regimen, in regard to calcium, influences the amount of calcium retained subsequently has been given by Fairbanks and Mitchell ('36). These authors found that the amount of calcium retained by rats during a given period varied inversely with the level of calcium in the ration fed previous to that period. They concluded that ". . . . the rate of calcium retention by growing animals under conditions of adequate nutrition measures the requirement of calcium only when the calcium stores have been saturated by appropriate pre-feeding." These findings point definitely to the necessity for careful preparation of all prospective subjects to be used in studies on calcium requirement. "Otherwise," in the words of the above investigators, "the observed calcium retentions will be greater than the day-to-day requirements for calcium."

Surprising fluctuations in the amount of calcium retained during several consecutive metabolic periods by growing children on a constant intake of calcium have been reported by Porter-Levin ('33–'34), by Hunscher, Hummel and Macy ('36–'37), and by Bonner et al. ('38). On analyzing her data, Porter-Levin ('33–'34) found that from 15 to 21 consecutive days were required to cover the range of variations in retentions of her subjects. In view of this fact, it would seem that much of the reported data based on short collection periods must necessarily be re-evaluated.

The purpose of the present study was to determine the maximum rate of calcium retention in well-nourished children (this measures the day-to-day calcium requirements for growth) and also to find the minimum daily intake of dietary calcium which would support maximum retention. At the beginning of the experiment, an attempt was made to saturate the body tissues with calcium by feeding generous quantities over a comparatively long period of time. It was hoped that this procedure would tend to minimize the influence of the previous regimens on the subsequent calcium metabolism of the subjects. The experimental periods which followed this period of preparation were sufficiently long to show the fluctuations characteristic of the individual and to obtain a reliable mean value for the child's requirement.

TABLE 1

The ages, weights and heights of the subjects and the comparisons of these with Grandprey's ('33) standards

SUBJECT	AGE[1]		WEIGHT[1]	HEIGHT[1]	LOCUS OF MEASUREMENTS		
	Years	Months			Weight—Age	Height—Age	Weight—height
			kg	*cm.*	%	%	%
B	3	9	14.1	93.5	65	25	Above 90
M	3	11	13.4	98.5	30	60	30
J	5	2	17.0	100.2	60	25	Above 90
P	5	8	17.4	110.5	50	70	65
S	5	11	18.2	105.4	75	10	Above 90

[1] Taken at seventeenth week of experiment.

EXPERIMENTAL

Subjects. Five healthy, pre-school girls, whose ages, heights, weights and the comparisons thereof with Grandprey's ('33) modifications of Woodbury's standards are given in table 1, were selected for the study.

The basal diet. The basal dietary was generous in all of the known necessary nutrients except calcium. Seven different groups of foods were selected (one for each day of the week), and these were fed weekly throughout the 32 weeks of the experiment. These foods are listed in table 2. A smaller

TABLE 2

Constituents of the basal diet for each day of the week

WEDNESDAY		THURSDAY		FRIDAY		SATURDAY		SUNDAY		MONDAY		TUESDAY	
Article of food	Weight	Article of food	Weight	Article of food	Weight	Article of food	Weight	Article of food	Weight	Article of food	Weight	Article of food	Weight
	gm.		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*
Apple	100	Pears	50	Peaches	50	Banana	70	Grapefruit	40	Prunes	25	Apricots	20
Lemon		Apple	75	Pineapple	50	Apple	75	Grapefruit		Pineapple	25	Prunes	15
juice	45	Grapefruit	40	Prunes	25	Carrots	20	juice	10	Banana	70	Pears	75
Fruit		Grapefruit		Tomato		Beans	30	Pear	50	Tomato		Beans	
juices	100	juice	10	juice	100	Potato	75	Pear juice	10	juice	50	(green)	20
Peaches	50	Carrots	10	Lettuce	10	Oatmeal	15	Banana	50	Cabbage	25	Potato	100
Tomato		Cabbage	25	Potato	50	Beef	60	Potato	100	Potato	100	Farina	25
juice	100	Potato	50	Spaghetti	15	Egg white	68	Sweet		Rice	20	Beef	60
Potato	50	Cornmeal	20	Rice	15	Egg yolk	16	potato	75	Tapioca	10	Egg white	68
Oatmeal	10	Macaroni	15	Tuna fish	60			Farina	20	Beef	60	Egg yolk	16
Rice	10	Dried beef	40	Egg white	68			Chicken	60	Egg white	68		
Spaghetti	15	Egg white	68	Egg yolk	16			Chicken		Egg yolk	16		
Liver	60	Egg yolk	16					broth	100				
Egg white	68							Egg white	68				
Egg yolk	16							Egg yolk	16				

quantity of vegetables than is generally recommended for children of this age was given in order that the diet might be low in calcium and accurately sampled for analytical purposes. Substances which were added to this diet, in order to compensate for the lack of vegetables or to make possible any necessary changes in the calcium and nitrogen intakes, are shown in table 3. In addition to these foods and concentrates, all of which were fed in definite daily quantities, each child ate ad libitum of bread (from which the crusts had been removed), butterfat, C.P. sugar and jelly in quantities sufficient to meet her individual caloric demands. A small variable portion of cornstarch was used in cooking. Redistilled water was used for cooking and drinking purposes as well as for the rinsing of dishes.

TABLE 3

Daily supplements to the basal diet

PERIOD	CALCIUM AS MILK SOLIDS	CALCIUM AS di-CALCIUM PHOSPHATE	CASEIN	IRON AS FERRIC CHLORIDE	TIKI-TIKI	VITAMIN D AS COD LIVER OIL	ASCORBIC ACID
	mg.	*mg.*	*gm*	*mg.*	*cc.*	*Int. units*	*mg.*
I	258	1466	10	15	5	360	100
II	258	....	10	15	..	360–1000[1]	100
III	516	....	3	15	5	1000	100
IV	774	....	3	15	5	1000	100

[1] At the twenty-first week the cod liver oil intake was increased from 1 teaspoonful to 3 daily.

The calcium level. As is indicated in table 3, the calcium was given as a supplement at four different levels during the experiment. For the first 15 weeks (period 1) generous amounts of di-calcium phosphate were fed. Following this interval, the calcium intake was decreased to approximately 370 mg. daily during the 8 weeks of period II. During periods III and IV, the level was raised successively to approximate averages of 615 and 880 mg., respectively.

Preparation of material for analysis. A 7-day metabolic period was selected. Carmine (350 mg.) was fed as a marker at the beginning of each weekly period. The total urinary and fecal outputs were collected for each consecutive week with the exception of the eleventh, twelfth and thirteenth weeks.

In weighing the foods for each meal, a portion identical with that to be served was saved; all such samples for the week were combined for analysis. The basal food, the bread and the stools were prepared for analysis according to the method of Stearns ('28–'29). The materials were allowed to digest in hydrochloric acid (1 : 5 dilution) until they could be strained through a 1 mm.-pore Büchner funnel. The small amount of indigestible fiber in both foods and stools was ashed separately and added to the corresponding digest. The strained material was then made to volume, and aliquots were withdrawn by means of special pipettes which had been prepared for use by breaking the tip (in order to increase the diameter of the bore) and re-standardized. Aliquots of the week's collection of urine were analyzed separately. The dish washings, obtained by cleaning the dishes with a rubber policeman and redistilled water, were also analyzed for the calcium content. They were evaporated to a small volume and digested with an acid-digestion mixture (1 part HNO_3 to 1 part H_2SO_4). The digest then was made to volume.

Analysis for calcium. The samples were evaporated to dryness over a steam bath, ashed in a muffle furnace at 600°C. and treated for the removal of silicates according to Frear and Kahlenberg's method ('33). Calcium then was determined by McCrudden's method ('11–'12). Di-brom cresol purple was used as an indicator, and the calcium was titrated with a standard solution of potassium permanganate.

RESULTS

The data for the calcium intake and balance for each subject during the four periods are given in table 4. The weekly balances for the five subjects (expressed as the daily average), as well as the average of the five values, are shown graphically in figure 1.

Calcium metabolism at a level of 1815 mg. daily (period I). The daily intakes of calcium during this period varied from 1804 to 1835 mg. for the five children and averaged 1815 mg.

As seen in table 3, the calcium was derived chiefly from di-calcium phosphate (1466 mg. daily). An additional quantity of calcium (averaging 100 mg.) was supplied by the basal food, and 258 mg. were contributed by milk solids.[2] The latter was equivalent to approximately 220 cc. of fluid milk.

The calcium balance of the individual subjects varied considerably from week to week. This variation may have been due in part to the inadequacy of the carmine marker to insure a complete separation of the feces, especially since the feces contained large amounts of calcium. The individual average values for the retention of calcium during the 10 weeks for which analyses were available were 121, 118, 151, 145 and 94, for subjects B, M, J, P and S, respectively, with a mean of 126 mg. daily.

TABLE 4

Daily calcium intake and retention for subjects

PERIOD		CALCIUM INTAKE (AVERAGE)	CALCIUM RETENTION					
Number	Weeks		Subject B	Subject M	Subject J	Subject P	Subject S	Average
		mg	*mg.*	*mg.*	*mg*	*mg.*	*mg.*	*mg.*
I	11	1800	121	118	151	145	94	126
II	8	370	72	50	94	80	84	76
III	5	615	117	84	145	142	124	122
IV	4	880	108	85	118	154	94	112

Calcium metabolism at a level of 370 mg. daily (*period II*). During this period the total daily intake of calcium was approximately 370 mg. The decrease in the calcium intake from that of period I was effected by the removal of the di-calcium phosphate supplement from the regimen for that period. No other change was made in the diet. The quantity of calcium given during this period was considerably less than that amount recommended for children of this age. However, it was from two to four times greater than the average retention during the period of liberal intake of calcium, and maximum retention could have been effected by every one of the subjects if 50% of the calcium had been available (Leitch, '37). But in each of the five subjects the retention of calcium decreased. As is seen in table 4 and figure 1, the mean values for the calcium balances during period II were 72, 50, 94, 80 and 84 mg. daily for subjects B, M, J, P and S, respectively, with a total average of 76 mg.

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[2] Skim milk powder, the calcium content of the various lots of which ranged from 0.0123 to 0.0133 mg. per gram.

Calcium metabolism at a level of 615 mg. daily (period III). The calcium intake during this period was increased over that in period II by the daily addition of a quantity of milk solids equivalent to 220 cc. of fluid milk to the diet of period II. It was desirable that the resultant level of intake would be slightly below (or barely equal to) the minimum amount required for maximum retention. The average retentions for each child were greater than during the previous period. The mean values were 117, 84, 145, 142 and 124 mg. for subjects B, M, J, P and S, respectively, with a total average of 122 mg. The values for subjects B, J and P were within 4% of the values obtained for period I. The value for subject B was considerably lower than and that for subject S was considerably higher than, the corresponding value for period I. The total averages for the two periods were almost identical.

Calcium metabolism at a level of 880 mg. daily (period IV). The calcium level during this period was obtained by adding milk solids equivalent to 220 cc. of fluid milk daily to the dietary fed during period III. The total milk consumption was approximately 660 cc. daily. This additional calcium was given in order to find whether or not it was possible to obtain a greater storage of calcium than was found during periods I and III. The values obtained for subjects B, M, J, P and S were 108, 85, 118, 154 and 94 mg., respectively, with an average of 112 mg. daily.

Fig. 1 The columns represent the average daily retention of calcium in milligrams for each week. The horizontal dotted lines represent the average daily retention for the various periods. The blank spaces indicate weeks during which analyses were not made, although the children were maintained on the diet. During the fifteenth week the children had a respiratory infection.

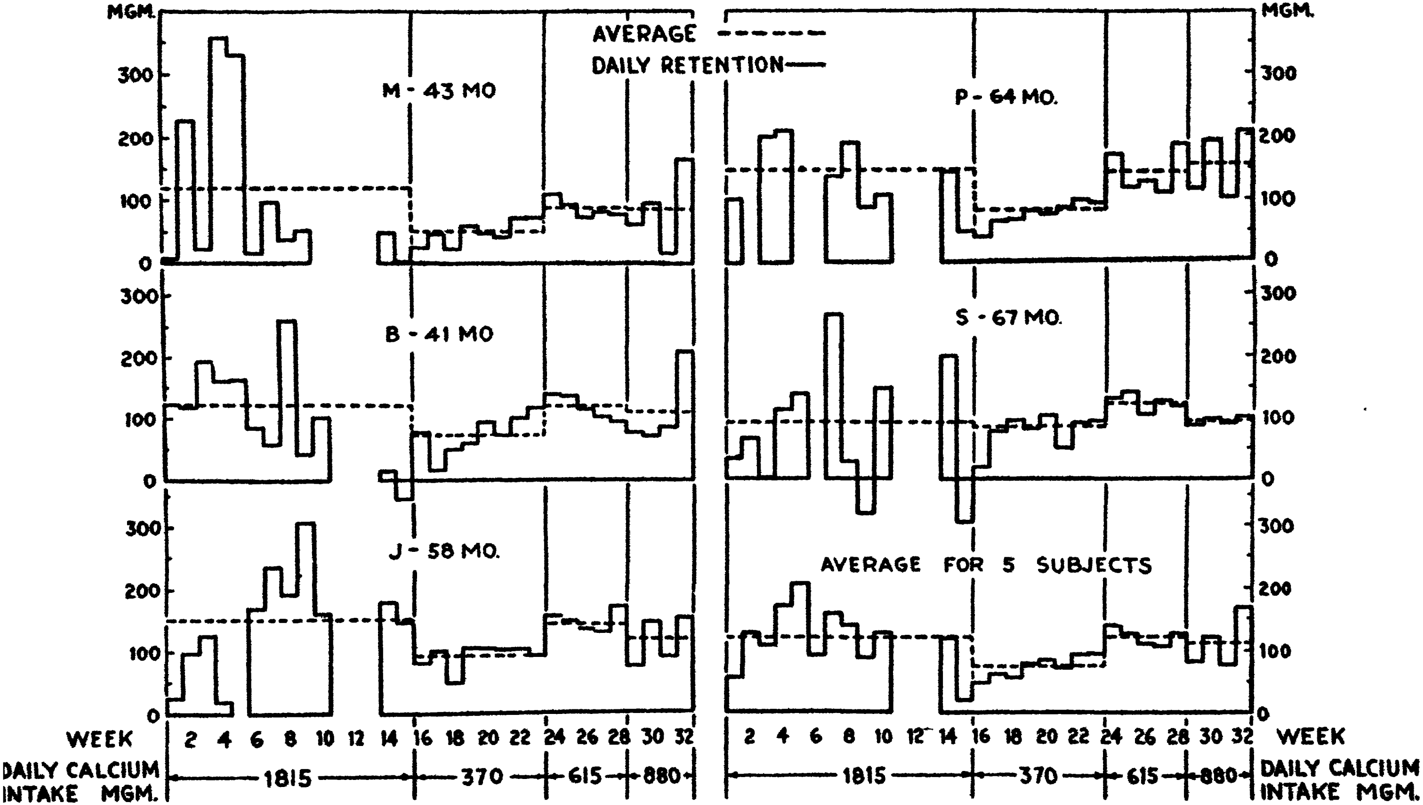
DAILY RETENTIONS OF CALCIUM AT DIFFERENT LEVELS OF INTAKE

Figure 1

DISCUSSION AND CONCLUSIONS

The data presented above will be discussed from the standpoints of the maximum quantities of calcium retained by the subjects and of the minimum amount of dietary calcium necessary to induce retentions of this magnitude.

In selecting values which would be representative of the day-by-day accretions of calcium in the bodies of the individual subjects under optimal dietary conditions, the data from period I were not considered because of the possibility that some of the calcium stored at this time was for the replenishment of depleted stores. During subsequent periods the maximum average amount retained by a given subject during a given period was considered to be characteristic of that individual's growth needs. These retentions were 117, 84, 145, 154 and 124 mg. of calcium daily (averaging 125 mg.), or 7.8, 6.2, 8.3, 8.8 and 6.5 mg. per kilogram (with an average of 7.5), for subjects B, M, J, P and S, respectively. None of these actual values approaches the average daily figure of 344 mg. recommended by Leitch ('37) as being necessary for skeletal growth of children. But the estimate of Leitch is obviously too high. A few authors have observed similar low retentions of calcium in children who had been fed presumably generous quantities of calcium but apparently have attached little significance to them. These authors have emphasized the maximum quantity retained even though the value was the result of only one analytical period. It is probable that a lower figure, especially if it is the average of several metabolic periods, more nearly represents the daily needs of the well-nourished child. The results of the present study would indicate that the daily growth requirement for these pre-school girls was approximately 125 mg. with a standard deviation of ±2.7 or, on the basis of body weight, 7.5 mg. ± 1.1 per kilogram.

The maximum average calcium retentions, ranging from 84 to 154 mg. daily, were attained on a calcium intake not greatly in excess of 600 mg. daily. On a 615 mg. intake, four of the subjects retained as much calcium as they did on the higher level (i.e., 880 mg.). Subject P retained slightly more calcium at this latter level than she did during any other period. The quantity of dietary calcium which could insure a retention of 154 mg. may be estimated on the basis of her individual ability to utilize dietary calcium. This figure would be approximately 665 mg. daily or 38 mg. per kilogram. For the remaining subjects, i.e., B, M, J, and S, the dietary requirements, calculated on the basis of weight, were 41, 44, 35 and 32 mg. per kilogram, respectively. None of these values fall within the range of 45 to 50 mg. per kilogram which Daniels et al. ('35) suggested as being sufficient for pre-school children.

During period III wherein maximum retentions were attained by four of the subjects, approximately five-sixths of the dietary calcium was contributed by milk solids. The fluid milk equivalent approximated 440 cc. daily. Subject P apparently needed slightly more—possibly as much as 490 cc. Obviously, if these children had been receiving a diet generous in vegetables (such as is usually recommended for children of their age), it is altogether probable that the addition of a pint of milk easily would have supplied enough calcium to meet the requirements of all five of them.

The contention that a pint of milk was sufficient for the calcium needs of these pre-school girls agrees with the conclusions of Daniels et al. ('34) whose subjects retained as much calcium when a pint of milk supplemented their basal food as they did when a quart of milk was used. However, it should be pointed out that their level of calcium intake, ranging from 750 to 875, was considerably higher than that required for maximum retention by the children reported in this paper. Although the quantity of milk supplements given in the two studies was approximately the same, the basal diet used by Daniels and co-workers was considerably richer in calcium.

The present study is the first reported in which an attempt has been made to determine the minimum amount of calcium which will insure maximum retention in well-nourished children over a comparatively long period of time. Obviously, the data may not apply necessarily to all pre-school children under all physiological and dietary conditions. Further discussion of the data will be withheld until a similar study on pre-school boys (which is now in progress) is finished.

Grateful acknowledgment is made to Dr. J. B. Gillespie of the Carle Hospital Clinic who examined the subjects from time to time and to Miss Helen King and Miss Frances Utterback who were responsible for the preparation of the children's food.

LITERATURE CITED

BONNER, P., F. C. HUMMEL, M. F. BATES, J. HORTON, H. A. HUNSCHER AND I. G. MACY 1938 The influence of a daily serving of spinach or its equivalent in oxalic acid upon the mineral utilization of children. J. Pediatrics, vol. 12, p. 188.

BURTON, H. B. 1929–1930 The influence of cereals upon the retention of calcium and phosphorus in children and adults. J. Biol. Chem., vol. 85, p. 405.

DANIELS, A. L., M. K. HUTTON, E. KNOTT, G. EVERSON AND O. WRIGHT 1934 Relation of ingestion of milk to calcium metabolism in children. Am. J. Dis. Child., vol. 34, p. 499.

DANIELS, A. L., M. K. HUTTON, E. M. KNOTT, O. E. WRIGHT AND M. FORMAN 1935 Calcium and phosphorus needs of preschool children. J. Nutrition, vol. 10, p. 373.

FAIRBANKS, B. W., AND H. H. MITCHELL 1936 The relation between calcium retention and the store of calcium in the body, with particular reference to the determination of calcium requirements. J. Nutrition, vol. 11, p. 551.

FREAR, D. E. H., AND O. J. KAHLENBERG 1933 A study of the accuracy of the McCrudden method for calcium and magnesium in biological materials. J. Biol. Chem., vol. 100, p. 85.

GRANDPREY, M. B. 1933 Range of variability in weight and height of children under six years of age. Child Dev., vol. 4, p. 26.

HUMMEL, F. C., H. A. HUNSCHER, M. F. BATES, P. BONNER AND I. G. MACY 1937 A consideration of the nutrition state in the metabolism of women during pregnancy. J. Nutrition, vol. 13, p. 263.

HUNSCHER, H. A., F. C. HUMMEL AND I. G. MACY 1936–1937 Variability of metabolic response of different children to a given intake of calcium. Proc. Soc. Biol. and Med., vol. 35, p. 189.

HUNSCHER, H. A., F. COPE, A. NOLL AND I. G. MACY 1933 Calcium and phosphorus storage in growing children. J. Biol. Chem., vol. 100, p. lv.

LEITCH, I. 1937 The determination of the calcium requirements of man. Nutr. Abs. and Rev., vol. 6, p. 553.

McCrudden, F. H., 1911–1912 The determination of calcium in the presence of magnesium and phosphates: The determination of calcium in urine. J. Biol. Chem., vol. 10, p. 187.

Petrunkina, A. 1934 Über die Bilanzen von Stickstoff, Kalk, Magnesium, Phosphor und Eisen der Kindern von 7–8 Jahren. Z. Kinderheilk, Bd. 56, S. 219.

Porter-Levin, T. 1932–1933 Calcium and phosphorus metabolism of normal preschool children. I. On diets containing plain and irradiated cereals. J. Am. Diet. Assoc., vol. 8, p. 482.

——— 1933–1934 II. Successive balance studies showing the range of variation in calcium and phosphorus storage. J. Am. Diet. Assoc., vol. 9, p. 22.

Sawyer, M., L. Bauman and F. Stevens 1918 Studies of acid production. II. The mineral loss during acidosis. J. Biol. Chem., vol. 33, p. 103.

Sherman, H. C., and E. Hawley 1922 Calcium and phosphorus metabolism in childhood. J. Biol. Chem., vol. 53, p. 375.

Stearns, G. 1928–1929 A rapid method for the preparation of fecal digests suitable for use in nitrogen and mineral analyses. J. Lab. and Clin. Med., vol. 14, p. 954.

Wang, C. C., M. Kaucher and M. Frank 1928 Metabolism of undernourished children. IV. Calcium metabolism. Am. J. Dis. Child., vol. 35, p. 856.

Wang, C. C., R. Kern and M. Kaucher 1930 Minimum requirement of calcium and phosphorus in children. Am. J. Dis. Child., vol. 39, p. 768.

Willard, A. C., and K. Blunt 1927 A comparison of evaporated with pasteurized milk as a source of calcium, phosphorus and nitrogen. J. Biol. Chem., vol. 75, p. 251.

THE UTILIZATION OF CARBOHYDRATE IN HUMAN UNDERNUTRITION[1,2]

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It has been shown repeatedly that the response of a normal animal to a test meal of glucose is profoundly altered by short periods of fasting or by a diet low in carbohydrate. This has been evident from studies of the blood sugar, oxidation experiments and, in a few instances, from analyses of tissues for their glycogen content after such test meals. Since an excellent review of the literature has recently been published by Chambers ('38) only those papers which have a significant bearing on the present studies will be discussed here.

In earlier work with the respiration chamber which has been described by Newburgh et al. ('37) it was observed that individuals who had been somewhat underfed in the preliminary periods often failed to oxidize all the carbohydrate of the diet in the experimental periods in the chamber even though they were still undernourished ('37). An attempt was therefore made to determine the conditions under which the normal human subject would exhibit diminished oxidation of carbohydrate, even though the diet was significantly below the caloric requirement.

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[2] A preliminary report of this work was presented at the meeting of the Society of Biological Chemists at Memphis, Tenn., April, 1937.

METHOD OF STUDY

The respiration chamber for human subjects was used and the gas analyses were carried out by the method of Carpenter ('33). Frequent analyses of out-door air were made and the chamber checked at intervals by means of alcohol checks. Table 1 gives the results for this chamber for the period of these experiments. The standard methods of indirect calorimetry were employed for the calculation of the heat production and the carbohydrate oxidized using the Lusk ('28) modification of the Zuntz-Shumburg table. The calories derived from carbohydrate were divided by 3.74, the caloric value of glucose, to determine the grams of carbohydrate oxidized. Two normal male subjects were used who lived in a special room in the laboratory. Both were students in the department of physical education and unusually intelligent and cooperative. In earlier studies made upon R.S., who was used for the greater number of the experiments, it was determined that he maintained his weight on a diet containing 3400 calories daily. The other subject, Van H., who was somewhat larger and of approximately the same age, undoubtedly needed a somewhat higher diet. All the diets used were the simple type employed in this laboratory consisting of bread, milk, butter, eggs, 40% cream, cane sugar and jelly, and were prepared in the laboratory. The preliminary feeding periods consisted of 2 or 3 days preceding the experimental day or days in the respiration chamber.

TABLE 1

Alcohol checks

DATE	ALCOHOL BURNED	R.Q	CARBON DIOXIDE			OXYGEN		
			Theoretical	Determined	Recovery	Theoretical	Determined	Recovery
	gm.		*liters*	*liters*	*%*	*liters*	*liters*	*%*
10/13/36	388.1	0.664	377.5	377.4	100.0	566.2	568.1	100.3
11/19/36	354.3	0.667	344.5	342.3	99.3	516.9	512.9	99.3
2/ 9/36	357.9	0.667	348.0	346.1	99.4	522.0	519.0	99.4
Averages		0.666			99.6			99.7

EXPERIMENTAL RESULTS

Four experiments were carried out on R.S. (nos. 1, 2, 3, 4, table 2) in which an attempt was made to deplete the glycogen stores by feeding a diet low in calories and containing varying amounts of carbohydrate. In the subsequent 24-hour period of observation in the chamber in each of these experiments, the carbohydrate content of the diet was approximately the same as in the preliminary period but the calories were reduced somewhat to keep them significantly below the heat production. The amount of carbohydrate fed varied from 52 to 133 gm. In none of these experiments, even though the caloric deficit was sometimes approximately 900 calories in each of the 2 preliminary days of feeding (exp. no. 1) and the carbohydrate as low as 52 gm. (exp. no. 2), could we demonstrate any impairment in the ability to oxidize the ingested carbohydrate.

In the next series of experiments (nos. 5, 6, 7, 8) the subject was placed on a preliminary diet of maintenance calories and containing a liberal supply of carbohydrate, 265 gm. After 3 days of this diet he entered the respiration chamber for the experimental period. In all of the experiments of this group, the first day of observation in the chamber the subject fasted, in order that we might measure the amount of the depletion of carbohydrate. The details of this group of experiments, in which the subject remained in the chamber 3 to 5 days may be found in table 2. It will be seen that in spite of the attempt to start the period of fasting with a sufficient and, we had hoped, an equal glycogen reserve, the carbohydrate oxidation on the day of fast varied from 84 (exp. 5) to 152 (exp. 8) gm. and did not vary directly with the heat production. This must be due to variations in the activity of the subject in the preliminary feeding periods so that we did not start each experiment with the same glycogen reserve. In experiment 8 we attempted to increase the stores by overfeeding in the prelimniary period and it will be observed that although the depletion on the fast day is the largest, no storage resulted on subsequent feeding. This is likewise true of

TABLE 2

SUBJECT AND EXP. NO.	DATE	PREPARATORY PERIOD		IN CHAMBER—24 HOURLY PERIODS						CARBOHYDRATE		
		Diet	Carbohydrate	Diet	Heat production	Caloric balance	Urinary N	Total CO_2	Total O_2	Ingested	Oxidized	Balance
		cal.	gm.	cal.	cal.	cal.	gm.	liters	liters	gm.	gm.	gm.
	1937											
R.S.	Feb. 18–19	1523	265									
1	20			1523	1875	— 352	13.4	317.3	396.1	133	137	— 4
	21			1523	1825	— 302	11.7	314.9	382.9	133	173	— 40
R.S.	Mar 29–31	1893	52									
2	Apr. 1			1755	1953	— 198	12.4	318.3	415.5	52	82	— 30
R.S.	Mar. 14–15	2526	107									
3	16			1518	1985	— 467	11.9	326.6	421.1	97	103	— 6
R.S.	Mar. 20–22	2526	107									
4	23			1720	2096	— 376	12.0	347.7	443.1	98	132	— 32
	Feb. 8–10	3500	265									
R.S.	11			Fast	1898	—1898	8.9	308.0	402.8	0	84	— 84
5	12			1378	1843	— 465	13.2	309.1	390.3	97	119	— 22
	13			1518	2011	— 493	11.2	332.5	424.6	133	120	+ 13
	1936											
	Nov. 27–28	3500	265									
R.S.	29			Fast	1935	—1935	9.5	324.8	407.3	0	146	—146
6	30			1518	2236	— 718	13.5	357.0	477.8	97	53	+ 44
	Dec. 1			1518	2073	— 555	11.5	343.4	438.6	97	124	— 27
	2			1518	2174	— 656	9.7	350.4	461.8	97	86	+ 11
	3			1518	2250	— 732	13.3	[illegible]	479.3	97	70	+ 27

	1937											
	Jan. 29–30	3500	265									
R.S. 7	31			Fast	1979	—1979	11.9	323.6	420.5	0	90	— 90
	Feb. 1			2139	2123	+ 16	11.7	347.3	450.4	160	104	+ 56
	2			2139	2025	+ 114	9.5	338.2	427.0	160	144	+ 16
	3			2139	2038	+ 101	9.1	345.6	427.5	160	177	— 17
	Feb. 25–26	3905	290									
R.S. 8	27			Fast	1706	—1706	8.0	290.1	357.7	0	152	—152
	28			1518	1970	— 452	11.8	323.1	418.4	97	95	+ 2
	1936											
	Dec. 9–10	3500	265									
	11			Fast	1942	—1942	8.1	325.2	408.4	0	146	—146
R.S. 9	12			Fast	1906	—1906	11.7	307.0	406.5	0	60	— 60
	13			2139	2090	+ 51	11.8	348.5	441.5	160	137	+ 23
	14			2139	2176	+ 37	8.7	356.8	462.6	160	126	+ 34
	15			2139	2144	+ 5	8.0	351.4	451.8	160	123	+ 37
R.S. 10	Oct. 24–25	1512	49									
	26			1512	2102	— 590	11.2	325.6	451.4	49	0	+ 49
	27			1512	2066	— 554	10.2	325.4	441.8	49	33	+ 16
	1937											
R.S. 11	June 8	0	0									
	9–10	1755	52									
				1728	1911	— 183	12.9	314.0	406.3	123	90	+ 33
	Apr. 1	0	0									
	2–3	1893	52									
Van H. 12	4			1893	2186	— 293	16.8	352.6	468.0	87	56	+ 31
	5	0	0									
	6			1882	2143	— 261	16.2	344.0	459.0	123	49	+ 74

experiment 5, while in 6 and 7 significant storage of glycogen resulted in the feeding periods subsequent to the fast and the amount of carbohydrate which failed to be oxidized is not directly related to either the calories ingested in the period after the fast or to the carbohydrate. In one case 82 gm. and in the other 72 gm. failed to be oxidized. In view of the limitations of the method one cannot attribute any significance to such a difference. In experiment 6 the caloric deficit in the feeding period averaged 702 calories and the carbohydrate ingested was 97 gm. while in experiment 7 the subject was slightly over-fed and ingested 160 gm. of carbohydrate.

One experiment, no. 9, was conducted in which the same preparation as above was used but was then followed by 2 days of fasting in the chamber and this in turn succeeded by 3 days of feeding with a diet containing 160 gm. of carbohydrate and exactly maintenance calories. Here 200 gm. of carbohydrate were oxidized in the 2 days of fast and storage of carbohydrate of approximately equal magnitude each day, accompanied resumption of feeding.

The demand of the organism for glycogen or the impairment of the ability to oxidize carbohydrate appeared therefore to be related to the degree of depletion of the previously existing stores. To accomplish sufficient depletion another program was tried—1 day of fast while the subject carried out his usual activity outside the chamber. This necessitated the expenditure of approximately 3400 calories and was therefore more drastic than the fast day in the chamber in which the maximal heat production was 1979 calories. This single day of fast was followed by 2 days of a diet containing 1512 calories and 49 gm. of carbohydrate (exp. 10). The 2 subsequent days were spent in the chamber and on both days the subject failed to oxidize the carbohydrate of his diet, oxidizing little or none the first day although his caloric deficit was 590 calories, and failing to oxidize but 16 gm. the second day. Exactly the same procedure was followed in experiment 11 except that the undernutrition was not quite so severe and only one 24-hour observation was made in the chamber. The diet ingested in the chamber contained 123 gm. of carbohydrate. The storage of carbohydrate in this case was only 33 gm., no greater than in the last experiment even though the carbohydrate available was greater and the caloric deficit smaller.

In the attempt to emphasize this relationship between the previous degree of depletion of the carbohydrate reserves and the failure to oxidize a given amount of ingested carbohydrate when the caloric deficit was decreased somewhat by confinement in the respiration chamber, a second subject, Van H., was subjected to a similar but somewhat more drastic regime. In experiment 12 he fasted for 1 day during which he carried out his usual program of activity outside the chamber. This was followed by 2 days on a diet containing 52 gm. of carbohydrate and 1893 calories. On the fourth day he was given 1893 calories containing 87 gm. of carbohydrate and observed in the respiration chamber. He failed to oxidize 31 gm. of the 87 ingested. The fifth day he again fasted outside the chamber and on the sixth day entered the chamber again where he received 1882 calories containing 123 gm. of carbohydrate. This time he failed to oxidize 74 gm. of the ingested carbohydrate. A possible error exists in the calculation of the carbohydrate oxidized in this last experiment. The nitrogen of the diet on the last experimental day was 13.8 gm. The subject excreted 16.2 gm. of urinary nitrogen for this 24-hour period. Some of the nitrogen may have represented delayed excretion of nitrogen resulting from the protein metabolism of the previous fast day. If, however, one calculates the carbohydrate oxidized using the nitrogen of the diet rather than the urinary nitrogen for this period, one still finds a failure of oxidation of 55 gm. The true value may lie between the two but the interpretation is not affected by such a difference.

DISCUSSION

In these experiments the failure to oxidize all the ingested carbohydrate might be due to 1) an impairment of the oxidative mechanism, 2) an increase in the rate of storage of glycogen over the normal, or 3) a failure to convert glucose to glycogen at a normal rate with subsequent failure of oxidation if glycogen synthesis is an obligatory step in the metabolism of carbohydrate. Chambers ('38) has summarized the literature and there seems to be little evidence that the organism in the early stages of carbohydrate depletion has any difficulty storing glycogen either in the muscles or liver. It should be pointed out, however, that most of the experiments deal with a glucose test meal, while in these experiments no carbohydrate was fed in this simple form. The carbohydrate of the diets used consisted of starch, lactose and sucrose. With regard to the first two possibilities, viz., decreased ability to oxidize carbohydrate and increased demand for glycogen by the tissues which have been previously depleted, it seems probable that both mechanisms are important. Cori and Cori ('28), in most carefully conducted experiments in which complete carbohydrate balances were made, found that rats which had been fasted 48 hours oxidized relatively less glucose and deposited relatively more glycogen than did those fasted 24 hours. This difference in deposition of glycogen was chiefly in the muscles. It may be that the impairment of the ability to oxidize glucose is associated with loss of glycogen from the muscles. In order to demonstrate failure of oxidation of carbohydrate or increased storage in these experiments, it was necessary to subject the individuals to a single fast day followed by a diet low in carbohydrate, or to 2 days of fast. Moderate depletion of the carbohydrate stores was not sufficient. The decreased ability to oxidize carbohydrate persisted for 24 hours or longer in some experiments, even though the carbohydrate was ingested in six equal meals at 4-hour intervals throughout the 24 hours, and even though the calories ingested were far below the energy requirement. The retention of carbohydrate under these conditions suggests the necessity for an optimal level of carbohydrate reserves for normal oxidation.

The interpretation of experiments of this type depend upon the validity of the method employed. The standard methods of calculation of indirect calorimetry were used for the calculation of the carbohydrate oxidized. It must be remembered that the respiratory quotient is a resultant of metabolic processes which may include interconversions of various foodstuffs as well as oxidative mechanisms. The method employed assumes it to be a pure combustion ratio or at least to such a large extent that other reactions influence it but little. If this be true, the calculation of carbohydrate oxidized is substantially correct. Adams and Poulton ('37) have shown by an analysis of experiments in which both direct heat has been measured in the calorimeter and heat production calculated from the respiratory exchange for simultaneous periods, that a tendency exists for the indirect heat to be higher at low respiratory quotients and lower at high quotients than the direct heat. Good agreement between the two methods occurs at respiratory quotients around 0.785. They believe that carbon dioxide bears a more direct relationship to heat production than does oxygen and postulate that carbohydrate and fat are burned at a fixed ratio in the body and therefore yield a fixed combustion ratio. Values below this mean conversion of fat to carbohydrate and above that the reaction carbohydrate to fat predominates. The evidence for such a theory is not convincing. However, in these experiments the respiratory quotient ranges close to that at which the calculated heat equals the direct heat measured in the calorimeter experiments and no large error should be present in calculating the heat production in the standard manner. No quotients below that of fat were noted.

Presumably here we are not concerned with the conversion of carbohydrate to fat since in the majority of experiments submaintenance diets were fed, and in the remainder, maintenance. If conversion of fat to carbohydrate occurs with the subsequent oxidation of the carbohydrate no effect on the respiratory quotient would be seen. If, however, the glucose newly formed were stored as glycogen, which might be the case under these conditions of previous depletion, the observed respiratory quotient might be lower than if it were a true combustion quotient. If such a conversion occurred its influence upon the 24-hour respiratory quotient would undoubtedly be small and therefore of but slight significance.

Further, since no conclusive evidence exists that such a conversion does occur, the standard methods of calculation seem to furnish the best evidence for the materials oxidized we can, at present, obtain.

SUMMARY AND CONCLUSIONS

The oxidation of carbohydrate by normal human subjects has been studied in the respiration chamber following various degrees of depletion of the carbohydrate reserves. The failure to oxidize all the ingested carbohydrate appears to be related to the degree of depletion and will result when the depletion is severe enough even though the calories of the diet are significantly below the maintenance requirements of the subject.

Impairment of oxidation occurred when the stores were greatly reduced by sharp reduction of the intake of calories and carbohydrate. One might expect that the organism under these circumstances would oxidize all the incoming carbohydrate for energy purposes. On the contrary, it stores considerable portions, even though the energy expenditure far exceeds the intake. Apparently it is more important to replenish the carbohydrate stores than to use the incoming carbohydrate for fuel.

LITERATURE CITED

ADAMS, T. W., AND E. P. POULTON 1937 Heat production in man. IV. Guy's, Hospital Reports, vol. 87, p. 107.

CARPENTER, T. M. 1933 Ein Apparat zur Analyse von Gasen aus Respirationskammern für Menschen und Tiere. Handbuch der biologischen Arbeitsmethoden, Abderhalden, E., Urban und Schwarzenberg. Berlin. Abt. LV, Teil 13, S. 593.

CHAMBERS, WILLIAM H. 1938 Undernutrition and carbohydrate metabolism. Physiol. Rev., vol. 18, p. 248.

CORI, CARL F., AND GERTY T. CORI 1928 The fate of sugar in the animal body. J. Biol. Chem., vol. 76, p. 755.

LUSK, GRAHAM 1928 The Science of Nutrition. W. B. Saunders Co., Philadelphia.

NEWBURGH, L. H., M. W. JOHNSTON, F. H. WILEY, J. M. SHELDON AND W. A. MURRILL 1937 A respiration chamber for use with human subjects. J. Nutrition, vol. 13, p. 193.

NEWBURGH, L. H., M. W. JOHNSTON, F. H. LASHMET AND J. M. SHELDON 1937 Further experiences with the measurement of heat production from insensible loss of weight. J. Nutrition, vol. 13, p. 203.

A NOTE ON THE VITAMIN B_1-SPARING ACTION OF FAT

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The evidence in the literature, that fat in some way 'spares' vitamin B_1, has been adequately reviewed by Cowgill ('34, '38). Two interpretations have been placed on this finding, a) thiamin is not required in the metabolism of fat and, when this foodstuff is metabolized in large quantities, the amount of vitamin required is less; b) thiamin is actually present in the fat. Even though interpretation (a) is accepted, the value of feeding fat is comparable to administering a certain amount of the vitamin. Accordingly, Cowgill ('34) has expressed this sparing action in terms of 'equivalence of vitamin,' which in the case of lard is 0.55 international units per gram (1.4 micrograms of thiamin chloride).

The idea that vitamin B_1 may be present in fat has received much support from investigations using fat solvents to prepare thiamin concentrates from naturally occurring sources. Myers and Voegtlin ('20) were able to extract the antineuritic substance from autolyzed yeast filtrate with olive oil and oleic acid. Because this substance was found to react like an organic base Van Veen ('32), Block and Cowgill ('32) were able to devise a procedure for the isolation of the vitamin based upon the principle that as the free organic base it is capable of extraction from alkaline aqueous solutions by organic solvents. Subsequently, Cook and Carroll ('36) found that fatty acid salts of oleic or caproic acid with vitamin B_1 can be made and that these salts may then be extracted by the same fatty acids. In view of these reports it is reasonable to believe that the so-called 'vitamin-sparing action of fat' may be due to the actual presence of thiamin in fat. The conclusive experiment for the justification of this point of view would be to isolate the vitamin from fat. Obviously such an experiment is not feasible because of the fact that the thiamin concentration in fat, if present at all, is exceedingly small. However, a procedure, which should be of equal value, is the analysis of fat for thiamin by a sensitive and specific method applicable to the determination of the vitamin in this foodstuff. For this reason we have applied our chemical method (Melnick and Field, '39 a) to the assay of lard for its thiamin content.

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[1] Upjohn Fellow in Clinical Research, 1937–1939.

EXPERIMENTAL

One hundred grams of lard,[2] to which 1 drop of concentrated sulfuric acid had been added, were melted on a steam bath. An equal volume of methyl alcohol was added and the mixture shaken vigorously in a separatory funnel. After the addition of 50 cc. of acidulated water and two ether extractions of the mixture, the aqueous phase was concentrated in vacuo until free from organic solvents, the pH adjusted to 4.5 and the diluted solution (100 cc. in volume) subjected to zeolite adsorption. No thiamin was present in the eluate. This experiment was repeated but this time 100 micrograms of thiamin chloride were added to the melted lard; 97 micrograms were recovered.

Because it is conceivable that the vitamin may exist to some extent as a free base at the pH of the body, and because as a free base it can form salts with fatty acids (Cook and Carroll, '36), tests were conducted on lard to determine whether any thiamin was present in such a form. If the vitamin were present as the fatty acid salt, simple aqueous extraction of the organic layer might have been unsatisfactory for the recovery of the thiamin. This belief is based upon our experience with phenol as an immiscible solvent for the extraction of the vitamin from aqueous solutions. Phenol has an extremely high solubility for vitamin B_1 (Greene and Black, '37). However, the quantitative recovery of the vitamin from the organic phase is exceedingly difficult. Thiamin was found to react with the solvent to form a phenolate which resists aqueous extraction despite the addition of large quantities of ether to the mixture; recoveries of the vitamin when such a procedure was employed varied from 20 to 50%. Only by use of a strong acid solution, such as 3.5 N H_2SO_4, in place of the aqueous phase previously used, were good recoveries possible, in the neighborhood of 90%. This principle was applied to the determination of thiamin in 100 gm. quantities of lard.

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[2] Obtained from Swift and Co., Chicago, Ill.

To the lard-methanol mixture, prepared as in the above tests, 4 volumes of ether were added and the solution extracted three times with 3.5 N H_2SO_4. The pooled acid extracts, totaling 100 cc. in volume, were carefully neutralized approximately at 5°C. to pH 2, concentrated in vacuo, the Na_2SO_4 precipitated with ethyl alcohol (80%) and removed by filtration, and finally the alcohol distilled in vacuo. The aqueous solution remaining was adjusted to pH 4.5, diluted to 100 cc. and subjected to zeolite adsorption (Melnick and Field, '39 a). In this case also no thiamin was found in the eluate. In order to test the adequacy of the procedures used, 100 micrograms of thiamin chloride were added to another 100-gm. sample of the lard and the test repeated. An 85% recovery of the added vitamin was obtained.

No analyses for the presence of the phosphoric esters of thiamin in lard were carried out because of the insolubility of such forms in organic solvents, even as the thiochrome (Kinnersley and Peters, '38) or diazo derivatives (Melnick and Field, '39 b) of the esters.

SUMMARY

Chemical analyses have indicated that lard contains no thiamin, justifying the conclusion that the vitamin B_1-sparing action of fat cannot be attributed to actual administration of the vitamin but to the decreased metabolic requirements for thiamin when the fat content of the diet is increased.

LITERATURE CITED

BLOCK, R. J., AND G. R. COWGILL 1932 The antineuritic vitamin. IV. The preparation of a highly potent concentrate. J. Biol. Chem., vol. 98, pp. 637–643.

COOK, C. A., AND R. H. CARROLL 1936 Accessory factors in rice polishings. I. Extraction of the antineuritic substance. Ind. and Eng. Chem., vol. 28, pp. 741–742.

COWGILL, G. R. 1934 Vitamin B Requirement of Man. xix, 265 pp. Yale University Press, New Haven, Conn.

——— 1938 The physiology of vitamin B_1. J. Am. Med. Assoc., vol. 110, pp. 805–812.

GREENE, R. D., AND A. BLACK 1937 Crystalline vitamin B_1 from natural sources. J. Am. Chem. Soc., vol. 59, pp. 1395–1399.

KINNERSLEY, H. W., AND R. A. PETERS 1938 Note upon the preparation of crude cocarboxylase from vitamin B_1 by yeast. Biochem. J., vol. 32, pp. 697–698.

MELNICK, D., AND H. FIELD, JR. 1939 a Chemical determination of vitamin B_1. II. Method for estimation of the thiamine content of biological materials with the diazotized p-amino acetophenone reagent. J. Biol. Chem., vol. 127, pp. 515–530.

——— 1939 b Chemical determination of vitamin B_1. III. Quantitative enzymic conversion of cocaboxylase (thiamine pyrophosphate) to the free vitamin. J. Biol. Chem., vol. 127, pp. 531–540.

MYERS, C. N., AND C. VOEGTLIN 1920 The chemical isolation of vitamins. J. Biol. Chem., vol. 42, pp. 199–205.

VAN VEEN, A. G. 1932 Die aus Hefe und Reiskleie gowonnen Krystallinischen Präparate des Vitamins B: I. Ztschr. physiol. Chem., Bd. 208, S. 125–128.

CHONDROITIN SULFURIC ACID AS A GROWTH FACTOR

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In the course of attempts to assay for riboflavin using chicks fed the ration proposed by Wilgus, Norris and Heuser ('35), it became apparent that this basal diet was deficient in other factors besides riboflavin. Many lots of chicks failed to grow on the positive control ration (riboflavin[1] supplemented), symptoms typical of anti-dermatosis factor deficiency often appeared in both positive and negative controls and the 'curly toe' type of paralysis described by Norris, Heuser, Wilgus and Ringrose ('31) was seen in some of the low-level positive controls. The addition of a rice-polish filtrate decreased the death loss, abolished the dermatosis and produced some improvement in growth. However, growth did not appear to be optimal even when the basal ration was supplemented with both riboflavin and rice-polish filtrate.

Since the present authors ('38) had previously found that chondroitin sulfuric acid (hereafter referred to as CSA) had a favorable effect on growth in some instances when added to diets which produce gizzard erosion in the chick, and because the properties of CSA bear some resemblance to those described by Jukes ('37 a) for the chick anti-dermatosis factor, it has been our purpose to determine the effect of adding CSA to the riboflavin assay basal ration.

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[1] The riboflavin used throughout these experiments was crystalline lactoflavin obtained from the Borden Company.

Chondroitin sulfuric acid (CSA) is the prosthetic group of chondromucoid (a protein of cartilage), and is composed of glucuronic acid, galactosamine to which an acetyl radical is joined, and sulfate groups. Crandall, Roberts and Gibbs ('32) and Crandall, Roberts and Snorf ('36) have shown that CSA had a systemic effect as evidenced by its therapeutic activity in migraine and the improved nutritional condition it produced in Eck fistula dogs. However, there is but one report suggesting that CSA (or one of its components) is of value in correcting dietary deficiencies. Bird and Oleson ('38) have reported that CSA prevented gizzard erosion, an observation which we have failed to confirm ('38) even though a large number of animals were studied on various erosion producing diets.

EXPERIMENTAL

In all of our present experiments involving chicks, day-old Barred Rocks were placed on the basal ration (or this ration supplemented as indicated) for a depletion period of 2 weeks. At the end of this time they were weighed, wing-banded and so placed in groups that average lot weights were comparable. About 30% were discarded as too light or too heavy, only chicks of medium weight being used. Each test lot contained between fifteen and twenty chicks. They were kept in temperature-controlled screen bottom cages in an air-conditioned room and were allowed free access to food and water.

The basal ration employed was that suggested for riboflavin assay by Wilgus, Norris and Heuser ('35). It consisted of: yellow corn 54, wheat middlings 20, vitamin-free casein 16, dextrin 4, cottonseed oil 3, steamed bone meal 2 and cod liver oil 1. Jukes ('37 b) modified this diet by supplying the anti-dermatosis factor from rice by-products. The rice polish filtrate used by us was prepared by extracting the polishings for 24 hours with 25% alcohol, acidifying and treating with fuller's earth to remove riboflavin. Completeness of riboflavin removal was checked photometrically.

In the first three groups of animals, the basal diet was given for 2 weeks, at which time supplements of rice-polish filtrate, rice-polish filtrate and riboflavin, and CSA and riboflavin were added. The results are given in table 1 and show that a much better growth and decreased death loss resulted from the CSA and riboflavin supplement as compared to the other two groups.

TABLE 1

Growth in chicks receiving supplements of rice polish filtrate and flavin as compared to CSA and flavin

TEST NO.	SUPPLEMENT	AVERAGE WEIGHT AT START (2 WEEKS)	AVERAGE WEIGHT AT 4 WEEKS	AVERAGE WEIGHT AT 6 WEEKS	GAIN	DEAD
		gm.	*gm.*	*gm.*	*gm.*	%
41S	Rice polish filtrate[1]	63	65	114	51	55
41T	Rice polish filtrate[1] and and 150 micrograms riboflavin per 100 gm. ration	65	104	158	93	33
41Z	3% CSA plus 150 micrograms riboflavin per 100 gm. ration	65	122	229	164	0

[1] Equivalent to 20% addition of rice polishings.

We next wished to determine whether CSA is identical with the chick anti-dermatosis factor. Dermatosis is readily produced by using a heated ration according to the method of Kline, Keenan, Elvehjem and Hart ('32). Accordingly, the corn, middlings, casein and dextrin were heated at 115°C. for 64 hours before incorporation in the basal ration. Three lots of chicks were selected after the previously outlined preliminary period of 2 weeks and were given supplements as in the first three groups. The results are given in table 2.

Dermatosis appeared as expected. The chicks receiving CSA and riboflavin showed a slightly better weight gain than those receiving riboflavin alone, but there was no alleviation of the dermatosis. Rice-polish filtrate produced the best gain and prevented dermatosis. It is evident that CSA is not the chick anti-dermatosis factor. The poor growth in all three of these groups is possibly due to destruction of amino acids in the casein by heat.

The next experiment was designed to determine the optimum amount of CSA for the chick when added to the basal ration. For this purpose, riboflavin (250 micrograms per 100 gm.) was added to the basal ration throughout the 2-week depletion period as well as during the remainder of the test. Purified CSA was used as a supplement in amounts up to 3% of the ration. The CSA was purified by hydrolysis of the commercial product with 2% sodium hydroxide for 24 hours, precipitation with slightly more than 1 volume of alcohol, solution in water, and reprecipitation with 6 volumes of glacial acetic acid. It was then washed thoroughly with alcohol and ether and drying completed in vacuo. The glucuronic acid content of the purified material was 36.0%; theory requires 37.6%. Growth-promoting effects of the several levels of CSA supplementation are recorded in table 3. The optimum amount appears to be about 2 to 3%; improved growth is produced by the lowest level (0.5%).

TABLE 2

CSA in the heated basal ration

TEST NO.	SUPPLEMENT	AVERAGE WEIGHT AT START (2 WEEKS)	AVERAGE WEIGHT AT 4 WEEKS	AVERAGE WEIGHT AT 6 WEEKS	GAIN	DEAD
		gm.	*gm.*	*gm.*	*gm.*	%
43J1	250 micrograms riboflavin per 100 gm. ration	53	52	52	—1[1]	80
43J2	250 micrograms riboflavin per 100 gm. ration plus 3% CSA	51	60	78	17[1]	66
43J3	250 micrograms riboflavin per 100 gm. ration plus rice polish filtrate[3]	51	66	99	48[2]	62

[1] Dermatosis present.

[2] No dermatosis.

[3] Equivalent to 20% addition of rice polishings.

The gizzards of all the chicks used in the tests listed in table 3 were carefully examined. When erosion was found, they were graded according to the following score: 1, no lesion; 2, slight erosion; 3, marked erosion and 4, severe erosion. The average scores for the various groups were 2.9 for the basal ration plus riboflavin but no CSA, and 3.2, 3.4, 3.6 and 3.4 for the 0.5, 1.0, 2.0 and 3.0% levels of CSA, respectively. This finding that the severity of the lesions is similar irrespective of the level of CSA fed confirms our previous observation ('38) that CSA is without effect on gizzard erosion.

TABLE 3

The effect of varying levels of CSA on chick growth

TEST NO.	CSA [1] ADDED	AVERAGE WEIGHT AT START (2 WEEKS)	AVERAGE WEIGHT AT 4 WEEKS	AVERAGE WEIGHT AT 6 WEEKS	GAIN	DEAD
	%	gm	gm.	gm.	gm.	%
44F	None	78	95	110	32	13
44G	0.5	80	105	152	72	0
44H	1.0	80	120	174	94	0
44I	2.0	80	130	191	111	0
44J	3.0	79	128	197	118	0

[1] Purified CSA added to basal ration containing 250 micrograms riboflavin per 100 gm.

TABLE 4

CSA supplementation of the basal ration using white rats[1] as test animals for a 7-week growth period

TEST NO	SUPPLEMENT	WEIGHT AT START	WEIGHT 7 WEEKS	AVERAGE GAIN	GRAMS FEED PER GRAM GAIN
		gm	gm.	gm.	
442	None	56	145	89	5.12
443	3% CSA	59	182	123	4.22
444	3% CSA plus 150 micrograms riboflavin per 100 gm. basal	55	180	125	4.25
445	150 micrograms riboflavin per 100 gm. basal	57	169	112	4.46

[1] Two male and four female rats, 4 weeks old, and evenly distributed by litter were used for each test lot.

To determine whether the growth-promoting effect of CSA is peculiar to the chick or whether it can be observed in mammalian forms as well, the experiments represented in table 4 were carried out with rats. The basal diet alone and supplemented by 3% CSA, 150 micrograms of riboflavin per 100 gm. of ration and 150 micrograms of riboflavin per 100 gm. of ration plus 3% CSA was fed to four groups. Each group was composed of two male and four female rats 4 weeks old.

It will be noted that when CSA was the only supplement the growth rate is much improved over that on the basal diet alone, and is as effective as the combination of CSA and riboflavin. It is questionable whether the difference between the group on riboflavin alone and those on CSA or CSA and riboflavin is significant.

DISCUSSION

It is evident from our data that CSA has a definite growth-promoting effect on the chick and the rat when added to the basal ration we have employed. Since the purified CSA gave no tests for protein or polypeptides and contained no known dietary essential as an impurity, the growth stimulation obtained must be attributed to the CSA molecule as such or to the glucuronic acid or galactosamine of which it is composed. Further experimentation is now in progress to determine whether either of these constituents is effective. It should be recalled that there is no known mechanism by which chondroitin may be split into its constituent parts. A sulfatase has been described which splits off sulfate radicals from CSA, but no enzyme has been found which is capable of hydrolyzing the remainder of the molecule into six carbon chain fragments.

We believe that CSA is not identical with any growth factors previously identified. The data show that it is not the anti-dermatosis principle and supports our previous contention ('38) that it is ineffective in gizzard erosion in the chick. CSA is capable of promoting growth when presumably adequate amounts of other known dietary essentials were present in the basal ration.

SUMMARY

Chondroitin sulfuric acid has been found to serve as a growth factor for the chick and rat. This material is not identical with the anti-dermatosis factor found in rice-polish filtrate. A 3% level of CSA was apparently optimal for growth in the basal ration used but did not prevent the occurrence of well-defined gizzard lesions.

LITERATURE CITED

Bird, H. R., and J. J. Oleson 1938 Effectiveness of chondroitin as the anti-gizzard erosion factor required by chicks. J. Biol. Chem., vol. 123, p. xi.

Crandall, L. A., Jr., G. M. Roberts and J. W. Gibbs 1932 The beneficial effect of mucin and chondroitin in experimental liver damage. Proc. Soc. Exp. Biol. and Med., vol. 29, p. 1082.

Crandall, L. A., Jr., G. M. Roberts and L. W. Snorf 1936 The use of chondroitin in idiopathic headache (including migraine). Am. J. Digestive Dis. Nutrition, vol. 3, p. 289.

Crandall, L. A., Jr., F. F. Chesley, R. E. Gray and H. E. Robinson 1938 Effect of chondroitin sulfuric acid on gizzard erosion and growth in chicks. J. Nutrition, vol. 17, p. 53.

Jukes, T. H. 1937 a Further observations on the assay, distribution, and properties of the filtrate factor. J. Biol. Chem., vol. 117, p. 11.

——— 1937 b The biological assay of lactoflavin with chicks. J. Nutrition, vol. 14, p. 223.

Kline, O. L., J. A. Keenan, C. A. Elvehjem and E. B. Hart 1932 The use of the chick in vitamin B_1 and B_2 studies. J. Biol. Chem., vol. 99, p. 295.

Norris, L. C., G. F. Heuser, H. S. Wilgus, Jr. and A. T. Ringrose 1931 The occurrence in chicks of a paralysis of nutritive origin. Poultry Sci., vol. 10, p. 93.

Wilgus, H. S., L. C. Norris and G. F. Heuser 1935 The relative protein efficiency and the relative vitamin G content of common protein supplements used in poultry rations. J. Agr. Res., vol. 51, p. 383.

EDITORIAL REVIEW

FACTORS INFLUENCING THE APPARENT ENERGETIC EFFICIENCY OF PRODUCTIVE PROCESSES IN FARM ANIMALS

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1. The influence of body size on apparent efficiency of milk production, egg production, growth and muscular work

In 1901 Jordan published in his book 'The Feeding of Animals' the following table:

TABLE 1

Efficiencies of productive transformations in farm animals (Jordan)

Animal and product	*Edible solids in pounds per 100 pounds of digestible organic matter in the ration*
Cow, milk	18.0
Hog, carcass	15.6
Calf, carcass	8.1
Fowl, egg	5.1
Fowl, carcass	4.2
Steer, carcass	2.8
Sheep, carcass	2.6

Jordan's table indicates that body size, species and nature of product are factors in the efficiency of productive processes. Popular belief supports this impression. The question of relative efficiency of milk production in large and small cows and (still smaller) goats is under lively debate. Similar discussions are popular as regards influence of size on other productive processes and breed associations advertise their favorite animals on the basis of their supposed superiorities.

Paper 179, Herman Frasch Foundation for Research in Agricultural Chemistry; paper 573, Missouri Agricultural Experiment Station, Department of Dairy Husbandry.

To the sponsor of the small animal the superiority of the (small) goat over the (large) cow is self evident. A 120-pound goat may attain the productive level of 15 pounds milk a day (1) whereas it appears altogether improbable that a 1200-pound cow could produce 150 pounds milk a day. Hence the conclusion that a small animal as a goat is superior as a milk producer to a large animal as a cow, and that perhaps the smaller the animal the higher the relative productivity. To clinch his argument, the sponsor of the small animal cites the rat, which in 3 weeks of lactation raises its newborn litter to a weight roughly equal to her own (2). As compared to the cow, or even to the goat, the milk production in the rat, in comparison to her body weight,[1] must be collosal.

The sponsor of large animals also finds plausible arguments to support his favorites. In the first place the basal metabolism per unit body weight is known to be less in large than in small animals (3), and therefore the maintenance cost per unit body weight is likely to be less in large than in small animals. If the milk production is proportional to maintenance cost, then the gross efficiency,[2] that is the amount of milk produced per unit feed consumed (rather than per unit body weight), is the same in large and in small animals; and if the gross efficiency and price per unit product are the same in large as in small animals, then the large animal is preferable because the management expenses (housing, milking, bookkeeping, etc.) are less in handling a few large, than many small, animals producing the same amount of milk in total (10).

Non-partisan considerations might perhaps lead to the expectation that there should be no difference in gross efficiency of productive processes in large and small animals. In the

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[1] Note that both sides overlook one aspect of the time element. A (small) rat or goat may produce more milk energy per unit body weight per day during a lactation period than a (large) cow, but what are the relative life-time productions per unit body weight, per unit basal metabolism, per unit maintenance expense in large and small animals? What are the relative lactation-period daily maxima and life-time productivities and gross efficiencies of small and large animals?

[2] $$\text{Gross efficiency} = \frac{\text{output energy (Cal.)}}{\text{input energy (Cal.)}} \qquad (1)$$

first place, if there were such size differences, the animal of the most efficient size would likely survive in the struggle for existence at the expense of the less efficient one. In the second place, if, as seems reasonable, there are no size differences in ability to assimilate and metabolize food, and it is not costlier to organize, for example, milk from nutrients in large than small animals, then the net efficiency [3] would be the same, and the gross efficiency [2] of the milk production might also be expected to be the same in large and small animals provided however that the milk-energy production and maintenance-energy cost change at the same relative rates with increasing body weight. The crucial question thus becomes: What is the relative influence of body weight on: 1) maintenance cost, and 2) productive level?

While there is general agreement on how basal metabolism varies with body weight (3) there is no agreement on how the feed cost of maintenance varies with body weight, because of the possibly variable activity increments with changing weight in different individuals and species. However, Kleiber (4) on the basis of extrapolation of limited data concluded that maintenance, productivity and basal metabolism all increase at the same relative rate, namely with the 3/4 power of body weight, and that consequently gross energetic efficiency of transformation is independent of body weight.

The lively interest among animal husbandmen and others concerning the influence of body size on productivity and efficiency, the practical importance and theoretical interest in the problem of efficiency of biologic transformations, and the contrast in the prevailing opinions on the problem led us to investigate it on: milk production in small and large dairy cows (5) [a problem pioneered by Gaines (6)], dairy goats (1), and rats (2); egg production in large and small fowls (7); muscular work in large and small horses and men (8); growth in small and large species of animals (5, 7); wool production in small and large sheep (9); lamb production in small and large sheep. While the results of these investigations are altogether tentative, they may be of interest in this connection.

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[3] Net efficiency $= \dfrac{\text{output energy}}{\text{input energy less maintenance energy}}$ (2)

The essential results on milk and egg production are summarized in table 2, which shows that within the limits of expected biologic variability, and experimental and computational errors: 1) the ratio of milk energy produced (in naturally high-milking animals) to digestible feed energy consumed (that is, gross energetic efficiency [2] of milk production) is not only independent of live weight within the species, but even between species; 2) the ratio of milk energy produced to basal metabolism energy is independent of live weight, from which it follows that 3) the ratio of total maintenance energy cost to basal energy metabolism is independent of live weight or even of species—it is (within the aforenoted limits of variability) the same in cows, rats and goats. We may thus conclude this discussion on milk-production efficiency as influenced by body size with the very important tentative generalization that maintenance-energy basal-metabolism energy and milk-production energy all tend to increase at the same relative rate with increasing body weight, and the gross energetic efficiency of milk production tends to be independent of body weight as such or even of species as such.

As previously noted and as shown in table 2, the intensity of milk production measured by the ratio of milk energy to body weight is very much greater in small than large animals (the ratio milk-calories produced per day to body weight in kilogram is of the order of 200 in rats and only 25 in dairy cows), but there are similar relative differences in the intensity of metabolic and consequently probably in maintenance energy expenditure, so that changing maintenance energy and milk energy with increasing body size just compensate, with the net result that the gross efficiency is the same in large and small animals despite the fact that per unit body weight milk-energy production is very much greater in small than large animals.[4]

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[4] It should be noted, however, that while energetic efficiency (a ratio) may not depend on body weight, monetary profit (a difference) is likely to. If gross efficiency and price of milk are the same, the larger the dairy cow, for example, the smaller the overhead cost of management per unit product and consequently the greater the profit (10).

TABLE 2

Gross energetic efficiency (with respect to total digestible nutrients consumed) of milk production in dairy cows, white rats and dairy goats (and related data); also egg production in fowls

	MILK PRODUCTION						EGG PRODUCTION	
	Dairy cows (5, 10)			White rats by methods A and B (2)		Goats (1)	Chickens (7)	
	'Ordinary' (superior) all breeds	'Extraordinary'		Arithmetic average of all litters			Ordinary fowls	Champion fowls
		Champion Holstein	Champion Jersey	A	B			
Number of cows, rat litters, goats, chickens	368	1	1	7	5	7	478	1
Body weight, kg.	513	771	318	0.291	0.295	42.5	1.7	1.8
Average milk (or egg) production Cal./day (length lactation period ranged in cows from ¼ to 1 year; in rats between 10 to 18 days lactation inclusive on 4- to 9-rat litters; goats from 11 to 25 weeks)	11,440	34,000	24,140	71.5	60.1	2114	49.4 Cal. egg/day	90 Cal. egg
Gross energetic efficiency of egg or milk production with respect to TDN energy, %	31	44	48	48	44	34.9 (32.0–40.5)	16	27
Estimated basal metabolism, Cal./day	6,700	9,000	4,700	29	29	1100	104	108
Ratio basal metabolism Cal./day to body weight, kg.	13	11.7	12.3	99	99	26	61	60
Ratio milk or egg Cal. to basal metabolism Cal.	1.7	3.8	5.1	2.1	2.1	1.9	0.48	0.83
Ratio milk or egg Cal. to body weight, kg.	22	44	63	207	149	49.8	29	50
$(\text{Wt.})^{0.73}$	95.1	128	67.1	0.406	0.411	15.4	1.473	1.536
Ratio milk or egg Cal. to $(\text{Wt. Kg.})^{0.73}$	120	266	360	148	147	136.9	33.5	58.6

A comparison of the productivities and efficiencies of egg (7) and milk (5) production in the right column of table 2 shows that for roughly comparable producers, the gross energetic efficiency[2] (16%) of egg production is about one-half that for milk production (31 to 35%). However, the net efficiency[3] (not counting maintenance cost) is of the same order for egg and milk production (the net efficiency with respect to TDN[5] energy consumption is from 60% to 70% for both milk and egg production (5, 7) while the top gross efficiency is shown in table 2 to be of the order of 27% for eggs and 48% for milk production).

The fact that net efficiency is probably the same in milk and egg production means that the mammary gland produces milk from TDN[5] with the same efficiency as the ovary-oviduct system produces egg. The fact that the gross efficiency[2] is less for egg than for milk production means that in comparison to maintenance expense less egg energy is produced in fowls than milk energy in cows. The possible explanation for the lower egg-energy production in comparison to maintenance cost than of milk-energy production, may be due to: 1) greater structural complexity in egg than milk (7); 2) as a result of 1) more biologically equivalent time (11) is taken to produce unit egg energy than unit milk energy, with consequently greater expenditure of overhead maintenance cost per calorie of egg than milk.

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[5] In this review the output energy is assumed to be the heat of combustion of the product, as milk, egg, meat; the input energy is assumed to be the metabolizable energy in the apparent total digestible nutrients consumed. 'Total digestible nutrients' is abbreviated in the literature to TDN, which equals to digestible carbohydrates + digestible protein + digestible fat × 2.25. It is also assumed that the thermal equivalent of the metabolizable energy is 4 Cal. per gram TDN or 1800 Cal. per pound TDN. The thermal values as reported in the literature range from about 1600 Cal. to about 1800 Cal. per pound TDN or from about 3.6 to 4.0 Cal. per gram TDN. The interested reader is referred for details to L. A. Maynard's 'Animal Nutrition,' McGraw-Hill, 1937; Armsby's 'Nutrition of Farm Animals' ('17), p. 650; Forbes and Kriss, Proc. Soc. Animal Production, 1931, p. 113; Christensen and Hopper, J. Agr. Res., Oct. 1, 1938. The efficiency computed on the assumption that 1 gm. TDN = 4 Cal. when multiplied by

becomes efficiency on basis that 1 gm. TDN = 3.6 Cal., and so on for other reference base values. It is thus easy to change efficiency from the basis of one to another thermal equivalent of TDN.

[2, 3, 5] See footnotes 2, 3 and 5 previously cited.

As regards growth, the problem of its gross efficiency[2] is complicated by the age factor. Thus according to Tangl, Glaser, Farkas, Bohr and Fauriet-Fremiet, among others, the gross efficiency of embryonic growth is of the order of 60 to 80% (12). According to Rubner and Lusk (13) the gross efficiency of early postnatal growth is (except in man) of the order of 34%. A part of this difference in result may be due to differences in muscular exercise, environmental temperature and nature and form of dietary energy. But most of the difference is due to increasing body weight and decreasing growth rate. The older the animal and the greater its size, the greater the maintenance expense of the body in comparison to weight gain. The over-all or gross energetic efficiency of a living converter is thus dependent on two factors: 1) maintenance cost which in turn depends on the size of the animal; and 2) speed of the converting process which depends on physiologic age (11) and may be dependent on the structural complexity of the product. Differences in gross efficiency of biologic transformations in productive processes—as egg or milk production, or growth—can usually be traced to these determining factors. Thus egg production is less efficient energetically than early growth, because whereas egg production includes the overhead maintenance cost of the fowl that produces the egg, growth of the chick does not include the overhead maintenance cost of its mother. With increasing age, however, a maintenance charge is built up by the increasing size of its body with a consequently decreased gross efficiency. Table 3 exemplifies the influence of age on estimated growth efficiency of small and large species of animals.

Table 3 shows that the over-all or gross efficiency of early postnatal growth at equivalent physiologic ages (11) is of the same order in large and small animals, and of the same order as of milk production, and higher than of egg production.

There is a decrease in gross growth efficiency with increasing age because in comparison to maintenance energy there is decreasing growth. The net efficiency of growth may not be affected by age, but gross efficiency must be.

Gross energetic efficiency of muscular exercise refers to the ratio of work performed to total energy expended as measured by oxygen consumption, rather than to the TDN-energy [5] consumption. That is, the work efficiencies do not include the cost of converting TDN to body fuel. Because of the difference in reference-base values, and in the nature of productive processes, the gross-efficiency values of muscular exercise are not directly comparable to gross-efficiency values for milk, or egg, or meat production. However, the maximum gross energetic efficiency of muscular work, which is near 25%, is the same in small and large horses and apparently also in man (8). So again we conclude that maintenance energy and production energy increase at the same relative rates with increasing body weight.

TABLE 3

Estimated gross efficiency of growth with respect to total digestible nutrients, TDN, consumed (5, 7)

POSTNATAL AGE (MONTHS FOR CATTLE, WEEKS FOR CHICKENS AND RATS)	GROSS EFFICIENCY, PER CENT						
	Chickens (Several groups under different dietary conditions)			Rats		Dairy cows	
	W. Leghorns	R. I. Reds	W. Leghorns	Males	Females	Holstein	Jersey
1	26	21	35	25	23	35	37
2	22	17	38	23	19	26	25
3	19	25	33	21	17	33	30
4	21	17	32	20	15	23	26
5	18	22	29	19	14	18	20
6	20	19	23	18	13	17	20
7	19	19	18			13	13
8	16	18	26			13	13

It thus seems permissible to conclude tentatively, that when other conditions (e.g., inheritance for maximum milk production, body fatness, food supply) are of the same order, the gross energetic efficiencies of milk production, egg production and muscular work tend to be independent of body weight and species as such. The gross energetic efficiency of growth is probably likewise independent of body weight and species as such but only for equivalent physiological ages (11).

2. The influence of endogenous and exogenous factors on apparent gross efficiency

Variation in productivities within a given species and weight class and consequently variations in gross efficiencies may be due to two types of influencing factors of equal interest to students of gross efficiency: 1) external or exogenous, as dietary, which are the major concern of students of nutrition and biochemistry, and 2) internal or endogenous, as circulatory, respiratory, excretory, which are the major concern of students of physiology. The catalysts (vitamins, hormones, enzymes) are of equal concern to students of nutrition, physiology and biochemistry, because: a) they affect all bodily transformations (compare influence of thyroxin or insulin on metabolic—nutritive—transformations); b) functionally they all serve as catalysts; c) depending on evolutionary circumstances the same substance may be vitamin (exogenous factor); hormone, or enzyme (endogenous factor). It is thus generally known that ascorbic acid is in some species (guinea pig) obtained from food, and is therefore a vitamin; in other species (rat) it may be produced in the body possibly as a hormone,[6] and it is in all cases an oxidation-reduction enzyme in the body. Similar relations exist between most other vitamins (14), enzymes, hormones, organizers (15), carcinogens (16), depending on the conditions of the field structure. There seems to be no functional or structural dividing line between at least some of the dietary and some of the hormonal factors.

Let us then discuss the problem of variability in gross efficiency as influenced by hormonic and dietary factors, illustrating it by the milk-production process. It is generally known that milk-production levels and consequently gross-efficiency levels are very much greater in dairy than beef cattle of the same body weight, or in dairy than mohair goats.

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[6] Ascorbic acid may possibly be produced in the digestive tract.

Body weight is obviously here eliminated as an influencing factor.

Broadly viewed, milk secretion is one of the many interdependent delicately synchronized mechanisms in the service of a single function, that of bridging the perilous gap between the dependent intra-uterine life and independent adult life. It has been definitely established (17) that the anterior lobe of the pituitary gland is the chief endogenous (hormonal) regulator of milk production. This gland is also the regulator of many other processes. Some investigators (17) maintain that milk secretion is the resultant of the action of a series of distinct hormones: 1) pituitary lactation hormone which regulates milk production by direct influence on the secreting cells of the mammary gland, and 2) metabolic hormones for protein, carbohydrate, fat, etc., each of which regulates milk production by its influence on a particular milk precursor. Other investigators maintain that the same biochemical entity (hormone) which stimulates milk secretion by mammary cells shows other regulating (hormonal) properties among which were reported (18) to be: 1) calorigenic in conjunction with the pituitary thyrotropic hormone; 2) calorigenic in thyroidectomized animals; 3) diabetogenic;[7] 4) crop-secretion stimulant in pigeons; 5) releaser of brooding instinct in fowls and maternal behavior in rats;[8] 6) sustainer of liver, pancreas, and intestines in pigeons; 7) participator in acceleration of growth. Indeed the lactation hormone is customarily assayed by its influence on crop growth (18) in pigeons of either sex rather than by its milk-stimulating property. The fact that the same substance which apparently stimulates mammary-gland cells to produce milk also induces maternal behavior gives a sort of philosophic functional unity to a diversity of phenomena, although similar philosophic unity could be deduced from the synchronized action of many distinct hormones.

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[7] However, Bergman and Turner reported separation of lactogenic and carbohydrate-metabolism anterior-pituitary hormones (Endocrinology, vol. 22, p. 619, 1938).

[8] However, Leblond and Nelson reported maternal behavior in hypophysectomized animals (Am. J. Physiol., vol. 20, p. 167, 1937).

There are similar perplexities as regards growth hormones. There may be a specific growth hormone as postulated by Evans (19), but Riddle (18, '37) reported support for the view that bodily growth is favored by various pituitary hormones. It seems reasonable that there may be many interlocking hormones acting as a harmonious unit in stimulating the process of growth and development.

Whichever the situation, it is undoubtedly possible to accelerate productive processes (lactation, growth, and so on) by hormone administration; and since increased level of productivity is normally associated with increased gross efficiency, one might perhaps hope to increase the gross efficiency of productive processes by hormone administration; or possibly by breeding animals for desirably high endocrine activity on the basis of knowledge of their hormonal constitution (17). Several questions suggest themselves in this connection: 1) Is immediate efficiency, as well as productive level, increased by hormone administration? 2) Can long-range efficiency (as contrasted to immediate efficiency) be increased by hormone administration? 3) Can long-range efficiency be increased by breeding for a special production-accelerating endocrine? Or would progress be more likely to follow selection for the desired long-time function which necessarily includes the participation of the entire organism, in the entire organic space-time field, including the aging process?

The organismic viewpoint leads to the inference that in the course of evolution the component parts of the body developed to function in organismic harmony, which would be disturbed with unfavorable after-effects, and therefore reduced long-range efficiency, either by dosing the animal with, or breeding for, one productive stimulator and leaving the remainder of the body unimproved. However, the influence of the time factor is not predictable. Thus the unfavorable after effects may not become marked until after the animal has reached the end of the desired productive period. This complex problem of immediate versus long-range efficiency needs careful formulation and investigation. Let us illustrate the possible interrelations between hormone administration and productive efficiency by the influence of thyroxin injection on lactation.

The increased milk production by thyroid feeding or thyroxin injection has been attributed in part to increased circulation rate (20). Thyroxin may also increase the speed of digestive and assimilative processes (21), which are so important for production. But in addition to speeding up circulation, digestion and assimilation, thyroxin administration speeds up the metabolism (maintenance item in equation 2)[9] of all systems. Thyroxin is a general metabolic accelerator that speeds the general tempo of life, of which milk production is only one manifestation; and it may be inferred from the results by M. Rubner, J. Loeb and R. Pearl (that acceleration of the rate of living accelerates the rate of senescence) that speeding up metabolism (the 'rate of living') by thyroid would accelerate senescence. Then, too, the thyroxin may conceivably overstimulate the vital organs, especially the circulatory system, thereby shortening the animal's life, as illustrated for example, by fatalities from auricular fibrillation in hyperthyroid individuals. Thus, even though thyroid administration increases the production level and might increase the immediate gross efficiency of milk production, it is not unlikely that it might also decrease the long-range gross efficiency by destroying the animal's usefulness prematurely.

Investigators of gross efficiency of transformation of energy-matter cannot overlook possible factors affecting longevity and fertility, because they enter into the reckoning of long-range efficiency. From the long-range viewpoint, it seems very important to pose such questions as: 1) Is extraordinarily rapid growth, or high productive level of any kind, brought about by dietary or/and hormonal methods compatible with maximum longevity and fertility? 2) What is the relation between successive increments of productive stimuli

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[9] See footnote 3. Equation (2) in slightly expanded form:

$$\text{Gross efficiency} = \frac{\text{milk energy}}{\text{milk energy} + \text{maintenance energy} + \text{other expenses}}$$

(e.g., successively larger administrations of dietary and hormonal factors) on longevity, fertility and long-range gross efficiency as contrasted to immediate productivity and efficiency? 3) May not the price paid for immediate success in achieving relatively high levels of productivity and gross efficiency be offset by unfavorable after effects with possible long-range failure?

We chose to discuss the influence of thyroxin on productivity and gross efficiency of lactation, a typical productive transformation, not only because the properties and effects of thyroxin are best known, but also because thyroxin may be administered in chemically pure form. It would be premature to discuss the influence on long-range gross efficiency of administration of other specific production-accelerating hormones, as the lactation hormone, because they have not yet been isolated. All that we know about the lactation hormone is that injection of anterior pituitary preparation exhibits, among other physiologic effects previously noted, initiation and maintenance and, in the absence of other limiting factors, possibly increase in milk production (17).

What has been said about hormone factors affecting milk-production efficiency appears to be applicable to growth efficiency; and what has been said about hormone factors may be said about dietary factors affecting productive processes. Growth has been enormously accelerated by injecting suspensions of whole anterior pituitary or its crude alkaline extracts (19). Growth has been likewise accelerated by dietary means (22). Indeed, during the duration of the experiment, the growth rate of the dietary-accelerated rats of Anderson and Smith or even of Mendel and Cannon (20) was higher than the growth rate of the hormone-accelerated rats of Evans (19). Unfortunately, Anderson and Smith did not have data on control rats of the contemporary stock fed an 'ordinary diet' at the same time, so that it is possible that the unusually high growth rate was not altogether the result of the unique dietary factors as such or of the appetizing nature of the diet which caused them to consume more feed.

Employing the paired-feeding method, some observers recorded accelerated growth as result of hormone administration (23, p. 399), and of vitamin, protein and mineral enrichment (23, pp. 400–404) on the same energy intake as normally growing rats. These results point to greater gross efficiency of growth in the accelerated than in normally growing rats; but in the absence of complete energy-balance data, or at least analyses of the composition of the gains, it may be argued that the superior growth rates under the influence of hormone administration or of special dietary supplements are confined to water, minerals and nitrogen rather than to total energy storage (23, pp. 400–402). There is need for investigating the influence of accelerated growth and other productivities as results of special dietary and hormonic factors not only on immediate efficiency but also on long-range efficiency which must be compatible with longevity, 'highest livability,' and fertility (23, pp. 383–392).

3. *The principle of optimal and limiting factors in efficiency*

'Normal' growth rate associated with 'normal' body composition, longevity and fertility is a resultant of interrelationships between innumerable intrinsic and extrinsic, endogenous and exogenous, factors; between 1) organizers, hormones, enzymes, responsiveness of the component tissues, and 2) food supply and other environmental factors. Genetic and non-genetic factors cooperate in the production of every trait. Thus (24) MacDowell observed striking differences in 'genetically-pure' litter-mate mice, living in the same cage on the same diet, and Newman, Freeman and Holzinger reported striking mental and physical differences in 'identical' (monovular) human twins living under different social-economic conditions.

A necessarily common characteristic of highly efficient animals whether the production be to meat, milk or eggs, is that the animals are high consumers of feed in comparison to maintenance. But what governs high feed consumption?

We have discussed the hormonal factors involved in stimulating production rate and consequently feed consumption.

As regards the nature of the diet, it is generally known that feed consumption is decreased and feed utilization is depressed when the diet is unbalanced with respect to certain constituents (23, p. 403).

Another factor affecting feed intake and gross growth efficiency is environmental temperature (25). This factor may be used to illustrate in a simple manner the principle of optimal and limiting factors. When temperature is too low gross efficiency is reduced because too great a portion of the feed is used to keep the animal warm. When temperature is too high, gross efficiency is reduced because at the upper belt of thermal neutrality the metabolic level is very low and the productive rate is correspondingly low. Between these two limits there is a point where the feed intake above maintenance, in comparison to maintenance, is a maximum and productive rate and gross efficiency are maximal. Similar reasoning is applicable when any endocrine activity, or muscular activity, or any other factor or function is too high or too low. It may be possible to represent the interrelations between the various components in the efficiency complex in some such manner as Henderson (26) represented interrelations between various components in the blood complex. The problem, in brief, is to map the limiting and optimal interrelationships in the time-space configuration which is the productive converter of energy-matter under investigation in order to furnish the background for intelligent thinking on nutritive processes in productive transformations (27).

LITERATURE CITED

(1) Univ. Missouri Agr. Exp. Sta. Res. Bull. 291, 1938 Growth, milk production, energy metabolism, and energetic efficiency of milk production in the dairy goat.

(2) Univ. Missouri Agr. Exp. Sta. Res. Bull. 285, 1938 A comparison of the amounts and energetic efficiencies of milk production in rat and dairy cow.

(3) Univ. Missouri Agr. Exp. Sta. Res. Bulls. 166, 1932 and 220, 1934 Basal metabolism, endogenous nitrogen, creatinine and neutral sulphur excretion as functions of body weight.

(4) Kleiber, M., 1933 Tiergrösse und Futterverwertung. Tierernährung, Bd. 5, S. 1; Ann. Rev. Bioc., vol. 6, p. 375, 1937.

(5) Univ. Missouri Agr. Exp. Sta. Res. Bull. 222, 1935, and Res. Bull. 238, 1936 Energetic efficiency of milk production and the influence of live weight thereon.

(6) Gaines, W. L., 1931 Size of cow and efficiency of milk production. J. Dairy Sci., vol. 14, p. 14.

(7) Univ. Missouri Agr. Exp. Sta. Res. Bull. 278, 1938 Energetic efficiency of egg production and the influence of live weight thereon.

(8) Univ. Missouri Agr. Exp. Sta. Res. Bulls. 209, 1934, 244, 1936, and Station Bull. 383, 1937 Energetic efficiency of horses, men and motors.

(9) Univ. Missouri Agr. Exp. Sta. Res. Bull. 287, 1938 Relation between body weight and amount of wool or feathers.

(10) Univ. Missouri Agr. Exp. Sta. Res. Bull. 239, 1936 Relation between monetary profit and energetic efficiency of milk production with special reference to the influence of live weight thereon.

(11) Brody, S., 1937 Relativity of physiologic time and physiologic weight. Growth, vol. 1, p. 60.

(12) Terroine, E., and R. Wurmser L'énergie de croissance. I. Le développement de l'Aspergillus niger. Bull. Soc. Chim. Biol., T. 4, p. 519, 1922; Joseph Needham, Chem. Embryology, Cambridge, 1931.

(13) Lusk, G., 1928 Elements of the Science of Nutrition. Philadelphia.

(14) Williams, R. R., 1938 The chemistry and biological significance of thiamin. Science, vol. 87, p. 559.

(15) Spemann, H., 1938 Embryonic development and induction. New Haven; Needham, J., Proc. Roy. Soc. Med., p. 1577, 1936.

(16) Fieser, L. F. Chemistry of natural products related to phenanthrene. New York, 1936; Bills, C. E., Physiology of sterols including vitamin D. Physiol. Rev., vol. 15, p. 1, 1935; Various authors in: Symposia on Quantitative Biology, vol. 5, 1937.

(17) For the most recent review see the chapter by C. W. Turner in the revised edition of *Sex and Internal Secretions* (edited by Edgar Allen, expected to appear in print late in 1938). Also Missouri Agr. Exp. Sta. Res. Bull. 259, 1937 (Gomez and Turner) and 266, 1937 (Reece and Turner).

(18) Riddle, O. Physiological responses to prolactin. Cold Spring Harbor Symposia on Quantitative Biology, vol. 5, p. 218, 1937 and Sci. Monthly, August, 1938.

(19) Evans, H. M., 1924 The function of the anterior hypophysis. Harvey Lecture.

(20) Graham, W. R., Jr. The effect of thyroidectomy and thyroid feeding on the milk secretion and milk fat production of cows. J. Nutrition, vol. 7, p. 407, 1934; the action of thyroxin on the milk and milk fat production of cows. Biochem. J., vol. 38, p. 1368; Herman, H. A., W. R. Graham, and C. W. Turner, The effect of thyroid and thyroxin on milk secretion in dairy cattle, Univ. Missouri Agr. Exp. Sta. Res. Bull. 275, 1938.

(21) Althausen, T. L., and M. Stockholm, 1938 Influence of the thyroid gland on absorption in the digestive tract. Am. J. Physiol., vol. 123, p. 577.

(22) Osborne, T. B., and L. B. Mendel The relation of the rate of growth to diet. I. J. Biol. Chem., vol. 69, p. 611, 1926; Mendel, L. B., and H. C. Cannon, The relation of the rate of growth to diet. II. J. Biol. Chem. vol. 75, p. 779, 1927; Smith, A. H., and F. C. Bing, Improved rate of growth of stock albino rats. J. Nutrition, vol. 1, p. 179, 1928–1929; Anderson, W. E., and A. H. Smith, Further observations of rapid growth of the albino rat. Am. J. Physiol., vol. 100, p. 511, 1932; Bryan, A. H., and D. W. Gaiser, The influence of diet and anterior pituitary growth hormone on the growth rate of adolescent rats. Am. J. Physiol., vol. 99, p. 379, 1931–1932; McCarty, R. G., The influence of diet on the growth of dairy heifers. Thesis for the degree of Master of Arts, University of Missouri, June 1938.

(23) Brody, S., 1935 Chapter on Nutrition, Ann. Rev. Bioch., vol. 4, p. 399.

(24) MacDowell, E. C., Scientific Monthly, vol. 42, 1936 as it relates to mice, and F. N. Freeman, Ibid., as it relates to men.

(25) Brody, S., 1934 Discussion of Kleiber's and of Giajas work. Ann. Rev. Bioch., vol. 3, p. 320.

(26) Henderson, L. J., 1928 Blood, a study in general physiology. New Haven.

(27) Mumford, F. B., 1938 Fifty years of Agricultural Experiment Station Work. Agr. Exp. Sta., University of Missouri, Columbia.

THE CALCIUM REQUIREMENT OF ADULT MAN AND THE UTILIZATION OF THE CALCIUM IN MILK AND IN CALCIUM GLUCONATE[1]

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The calcium requirement of the adult organism relates to the replacement of endogenous losses of calcium from the body, mainly through the kidneys and the alimentary tract. While the cause of these losses is quite obscure, their existence has been definitely established in many published experiments on human subjects. We may consider the endogenous loss of calcium per day as measuring the requirement for maintenance. It is undoubtedly dependent upon body size, but whether it varies directly with body weight, body surface or with some other power function of body weight is not at present known. However, until this question is settled the endogenous losses of calcium may be tentatively expressed to the kilogram of body weight.

The efficiency of dietary calcium in replacing endogenous losses may be expected to vary with the kinds of food materials contained in the diet. Hence the dietary requirement of calcium for maintenance will vary with different diets and will probably always be much greater in magnitude than the endogenous loss. In a compilation and analysis by Mitchell and Curzon ('39) of available information on the subject including 139 average observations on 107 human subjects, only eighteen of whom were males, it was found that the average endogenous loss of calcium was 3.1 mg. per day per kilogram of body weight, while the average intake of dietary calcium required for calcium equilibrium was 9.75 mg. per day per kilogram of body weight. Thus, slightly more than 3 gm. of dietary calcium is required on the average to replace an endogenous loss of 1 gm. But considerable disparity exists among the individual data here summarized, partly the result of metabolic differences among the various subjects studied, partly the result of the short experimental periods usually employed in human metabolism studies, and partly the result of the many different types of diets employed, containing different sources of calcium.

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[1] This investigation was aided materially by the donation of funds from the Graduate School of the University of Illinois, for which grateful acknowledgment is made.

It seems important to know the extent to which the calcium in human foods is utilized by the adult, particularly those foods that supply important amounts of calcium to the American diet. Most of the experimental evidence concerning the utilization of the calcium of foods by human subjects reviewed by Mitchell and Curzon ('38) is of comparative, not absolute, significance. In none of the experiments on adults there reviewed is it possible to compute from the reported data the per cent to which the dietary calcium was used in the body in the replacement of endogenous losses of calcium.

The experiment to be reported below was designed to measure the calcium excretion of an adult human on a low calcium diet, and to assess the value of the calcium in milk solids and in an inorganic salt, calcium gluconate, in replacing these losses and in maintaining a condition of calcium equilibrium. Since the human organism, perhaps to a greater extent than the lower animals, is subject to wide fluctuations in its utilization of dietary minerals, the experiment was planned to extend over much longer than the usual periods of time. A total of 168 days of experimental observation is included in this experiment.

PLAN OF EXPERIMENT

The subject of the experiment was one of the authors (F.R.S.), about 35 years of age, weighing normally about 85 kg., and 176.5 cm. high.

The plan of the experiment was to subsist throughout upon a low-calcium diet, upon which the daily loss of calcium in urine and feces would be determined. Then graded additions of calcium in the form of skim milk powder or of calcium gluconate were made to the diet and the calcium balance determined on each level in the attempt to find out how much calcium in the two forms would be required for calcium equilibrium. Such data would also permit the determination of the utilization of the calcium in each case by relating increments in calcium balance to increments in calcium intake.

The collection periods were of 4 days duration, though each experimental period of definite calcium intake lasted from 12 to 32 days. The feces of successive collection periods were separated with the aid of carmine markers. Each day samples of the foods consumed, in the proportions in which they were eaten, and equal in amount to one-fourth of the day's consumption, were collected, and every 4 days these samples were composited for analysis for calcium. Modifications of the McCrudden method were used in the analysis of food, feces and urine.

The low-calcium basal ration that was used throughout the experiment was as follows:

Breakfast	
Orange juice	100 cc.
Oatmeal	224 gm.
Lunch	
Bread	50 gm.
Bacon	48 gm.
Oatmeal cookies	60 gm.
Apples	180 gm.
Dinner	
Potatoes, baked	160 gm.
Beef	80 gm.
Beets	100 gm.
Prunes	120 gm.

The ratio of calcium to phosphorus in this day's menu was found by actual analysis to be 0.3 to 1. The day's intake of protein and energy in the basal diet amounted to about 64 gm. and 2627 calories, respectively, including the sugar and butter supplements given below.

Tea, made with distilled water, was taken with the noon and evening meals and all water drunk was distilled. During the day 94 gm. of sugar, partly in the form of candy, and 56 gm. of butter were also consumed. Vitamins A and D were provided daily in a vitamin capsule (Parke, Davis and Company) equivalent to 3 teaspoonfuls of cod liver oil. The skim milk powder supplements, ranging from 10 to 50 gm. daily, provided additional energy, as well as calcium, a circumstance that was considered favorable in view of the tendency for the body weight to decrease during the experimental periods. Following the intermission during the latter part of March and the first few days of April, and continuing throughout the calcium gluconate periods, the basal diet was supplemented with a further addition of 40 gm. of sugar daily in order to raise the energy intake to the level of the preceding periods when milk powder supplements were fed. The preparation of calcium gluconate used contained 9.084% of calcium.

The experiment consisted of forty-three 4-day periods, the sequence of which was interrupted only twice, from December 20th to January 6th and from March 23rd to April 7th. Throughout the period of experimental feeding the subject was in good health, and in fact felt better than usual.

EXPERIMENTAL RESULTS

The calcium balance data are summarized in table 1. The basal diet provided an average of 195 mg. of calcium daily, on which the average daily balances for the three basal periods were, in order, —99, —121 and —109 mg. daily, averaging —110 mg. The calcium excreted daily on this low-calcium diet averaged 304 mg., of which 68% was excreted by way of the intestinal tract. The close agreement of the metabolism

TABLE 1

The calcium metabolism data of the experiment

PERIOD	INCLUSIVE DATES	BODY WEIGHT	CALCIUM SUPPLEMENT CONSUMED DAILY	DAILY CALCIUM METABOLISM			
				Intake	Feces	Urine	Balance
		kg.		*gm.*	*gm.*	*gm.*	*gm.*
1[1]	Nov. 6–9	85.5	None	0.160	0.358	0.105	–0.303
2	Nov. 10–13		None	0.180	0.197	0.086	–0.103
3	Nov. 14–17		None	0.164	0.193	0.102	–0.131
4	Nov. 18–21	82.7	None	0.189	0.182	0.092	–0.085
5	Nov. 22–25		None	0.164	0.205	0.086	–0.127
6	Nov. 26–29		None	0.166	0.199	0.063	–0.096
7	Nov. 30–Dec. 3	80.8	None	0.228	0.204	0.070	–0.046
8	Dec. 4–7	81.0	None	0.198	0.226	0.072	–0.100
Averages		82.5		0.184	0.201	0.082	–0.099
9	Dec. 8–11	80.5	10 gm. milk solids	0.280	0.262	0.089	–0.071
10	Dec. 12–15		10 gm. milk solids	0.292	0.282	0.086	–0.076
11	Dec. 16–19	80.5	10 gm. milk solids	0.290	0.281	0.111	–0.102
Averages		80.5		0.287	0.275	0.095	–0.083
12[1]	Jan. 6–9		None	0 195	0.286	0.073	–0.164
13	Jan. 10–13		None	0.210	0.209	0.093	–0.092
14	Jan. 14–17		None	0.182	0.203	0.130	–0.151
Averages		. . .		0.196	0.206	0.111	–0.121
15	Jan. 18–21		40 gm. milk solids	0.692	0.575	0.140	–0.023
16	Jan. 22–25		40 gm. milk solids	0.709	0.584	0.127	–0.002
17	Jan. 26–29		40 gm. milk solids	0.716	0.650	0.118	–0.052
18	Jan. 30–Feb. 2	80.7	40 gm. milk solids	0.705	0.589	0.129	–0.013
19	Feb. 3–6	80.1	40 gm. milk solids	0.725	0.552	0.117	+0.056
Averages		80.4		0.709	0.590	0.126	–0.007
20	Feb. 7–10	80.3	50 gm. milk solids	0.832	0 755	0.189	–0.112
21	Feb. 11–14		50 gm. milk solids	0.856	0.652	0.163	+0.041
22	Feb. 15–18		50 gm. milk solids	0.856	0.751	0.143	–0.038
23	Feb. 19–22		50 gm. milk solids	0.850	0.697	0.136	+0.017
24	Feb. 23–26		50 gm. milk solids	0.868	0.677	0.144	+0.047
25	Feb. 27–Mar. 2		50 gm. milk solids	0.880	0.792	0.130	–0.042
Averages		80.3		0.857	0.721	0.151	–0.015
26	Mar. 3–6		40 gm. milk solids	0.718	0.582	0.126	+0.010
27	Mar. 7–10		40 gm. milk solids	0.726	0.609	0.121	–0.004
28	Mar. 11–14	79.8	40 gm. milk solids	0.745	0.605	0.126	+0.014
29	Mar. 15–18		40 gm. milk solids	0.721	0.634	0.096	–0.009
30	Mar. 19–22		40 gm. milk solids	0.758	0.647	0.147	–0.036
Averages				0.734	0.615	0.123	–0.004
31[1]	April 7–10	81.2	None	0.197	0.369	0.090	–0.262
32	April 11–14		None	0.209	0.214	0.098	–0.103
33	April 15–18	79.6	None	0.185	0.206	0.093	–0.114
34	April 19–22		None	0.196	0.215	0.106	–0.125
35	April 23–26	79.6	None	0.225	0.219	0.102	–0.096
Averages		80.1		0.204	0.213	0.100	–0.109
36	April 27–30		7.038 gm. Ca gluconate	0.816	0.640	0.160	+0.016
37	May 1–4		7.038 gm. Ca gluconate	0.884	0.600	0.186	+0.098
38	May 5–8	78.7	7.038 gm. Ca gluconate	0.796	0.577	0.172	+0.047
Averages		78.7		0.832	0.606	0.173	+0.053
39	May 9–12		5.304 gm. Ca gluconate		Collections lost		
40	May 13–16	78.9	5.304 gm. Ca gluconate	0.682	0.565	0.149	–0.032
41	May 17–20		5.304 gm. Ca gluconate	0.689	0.484	0.155	+0.050
42	May 21–24	78.2	5.304 gm. Ca gluconate	0.679	0.610	0.165	–0.096
43	May 25–28		5.304 gm. Ca gluconate	0.683	0.540	0.166	–0.023
Averages		78.5		0.683	0.550	0.159	–0.026

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[1] The data for these periods are not included in the averages.

data in the three periods (November 6th to December 7th, January 6th to 17th, and April 7th to 26th) of subsistence on the basal diet alone is significant.

Forty grams of skim milk powder daily, providing about 542 mg. of calcium (1.355%) was sufficient in addition to the 195 mg. of calcium in the basal diet, to maintain the subject in approximate equilibrum, the average balance over a 20-day period being —7 mg. daily and over a subsequent 20-day period, —4 mg. daily. In the intervening 24-day experimental period, a supplement of 50 gm. of milk powder was no more effective than 40 gm. in maintaining calcium equilibrium, indicating the sufficiency of the latter supplement for calcium equilibrium.

For the 12 days from April 27th to May 8th, a daily supplement of calcium gluconate providing 639 mg. of calcium promoted some calcium storage averaging 53 mg. per day. During the following 20 days, this supplement was decreased to provide only 482 mg. of calcium daily. The contemporary balances of calcium were generally negative and averaged —26 mg. for the last 16 days for which balances were determined. The results indicate that 639 mg. of calcium added to the basal diet as the gluconate salt were more than adequate for the maintenance of calcium equilibrium, while a supplement of 482 mg. was inadequate. If interpolation is permissible on the basis of the observed balances of calcium, it appears that 534 mg. of added calcium as the gluconate would have established calcium equilibrium. This estimate is practically identical with the quantity of milk calcium found to be adequate—or only very slightly inadequate—for equilibrium, i.e., 542 mg.

DISCUSSION

The equation that Mitchell and Curzon ('39) derived to describe the average relationship in adult human subjects between the output of calcium and its intake is:

$$y = 0.6826\,x + 3.0940$$

where y is the calcium output in milligrams per kilogram of body weight per day and x is the intake of calcium expressed in the same fashion. In the experiments reported above, the average body weight of the subject in the three experimental periods in which no calcium supplement was added to the basal diet was 81.1 kg. His average daily intake of calcium per kilogram of body weight was therefore $195 \div 81.1 = 2.40$ mg. His average output on the same basis was $304 \div 81.1 = 3.75$ mg. Placing the former value for x in the above equation, gives a value for $y = 4.73$ mg., about 26% higher than the observed output, 3.75 mg. per kilogram. This divergence from the average relationship is perhaps not surprising in view of the disparity existing among the data from which the equation was derived. The coefficient of x in this equation indicates an average utilization of 32% ($[1 - 0.6826] \times 100 = 31.74$) of the dietary calcium.

About 540 mg. of calcium either in skim milk powder or in calcium gluconate seemed sufficient to attain calcium equilibrium in this subject in addition to the 195 mg. of calcium in the basal foods. Equilibrium was thus attained on a total of 735 mg. of calcium, equivalent to about 9.2 mg. per kilogram of body weight, as compared with the average of 9.75 mg. derived by Mitchell and Curzon ('39) from available data in the literature.

The daily addition of 540 mg. of calcium in the form either of milk solids or of calcium gluconate to the basal diet changed the daily calcium balance from an average of —110 mg. to approximately 0. In other words 540 mg. of calcium in these forms were required to replace an endogenous loss of 110 mg., indicating a utilization of the dietary calcium of about 20%.

This is a much lower utilization of milk calcium, or of dietary calcium of any origin, than is frequently assumed. It is difficult to find in the literature, however, any precise determinations of calcium utilization, since the information sought and obtained has been comparative in significance; for example, the relative utilization of the calcium of milk and of carrots. Such evidence as the following seems worthy of citation. Mallon, Jordan and Johnson ('30) found that an intake of 9.6 mg. of calcium per kilogram of body weight daily, 94% of which was derived from milk, induced a small storage of calcium in one woman subject but a sizable loss of calcium in another. Mallon, Johnson and Darby ('32) reported that 9.3 mg. of calcium per kilogram of body weight per day of which 87 to 88% was supplied by milk was slightly inadequate for calcium equilibrium for two women subjects. In a study reported the following year ('33), the same investigators showed that a diet providing 9.8 and 10.5 mg. of calcium per kilogram of body weight daily, 88% of which was derived from milk, promoted a slight storage of the element in two female subjects. Using a group of ten college women as experimental subjects, Kramer, Potter and Gillum ('31) were unable to secure positive calcium balances on daily intakes of calcium ranging from 5.7 to 9.0 mg. per kilogram of body weight, 78 to 80% of the food calcium being derived from either milk or ice cream. These experimental results are consistent with the results reported in this paper, indicating a requirement of 9.2 mg. of calcium per day per kilogram of body weight for equilibrium, milk or calcium gluconate furnishing about 73% of the calcium consumed.

On the other hand, investigations by Adolph and Chen ('32), Rose and MacLeod ('23), Rose ('20) and Kramer, Latzke and Shaw ('28) indicate that diets providing daily from 8.2 to as low as 6.4 mg. of calcium per kilogram of body weight, supplied largely from milk, may promote calcium storage in adult human subjects for short periods of time, at least.

If these short-time calcium balance studies on adult subjects may be taken at their face value, they suggest that the utilization of calcium in any particular food, such as milk, is subject to considerable variation depending upon unknown factors, dietary or non-dietary or possibly both. It is not clear that the potential acidity or alkalinity of the diet, the proportion of fibrous materials in it, the proportion or character of the fat or mineral ratios are of preponderate importance at least when adequate amounts of vitamin D are available. The possibility of raising the utilization of dietary calcium in the adult by varying the above (or other) factors is an important problem in human nutrition.

Turning to the calcium metabolism of children, it appears that, while the infant may utilize milk calcium to the extent of 60% or so (Mitchell and Curzon, '39), older children do not do so well. Sherman and Hawley ('22) placed three children, varying in age from 4 to 12 years, upon graded supplements of milk added to a low calcium diet. Comparing successive periods in these experiments, in which the milk intake was increased or decreased at the rate of 250 cc. daily, it may be shown that the increments or decrements in calcium retention average only 24% of the simultaneous increments or decrements in intake. Unpublished experiments of Outhouse, Kinsman and others at the University of Illinois on eleven preschool children afford excellent evidence of the efficiency in the utilization of milk calcium. Measuring the utilization of milk calcium by relating increments in calcium retention to corresponding increments in intake of milk calcium within ranges of the latter not exceeding the requirements for maximum retention, the percentages of utilization varied from 14 to 25 and averaged 20, exactly the same as the value secured on an adult reported in this paper.

SUMMARY

The calcium balance of an adult human subject receiving a basal low-calcium diet with varying supplements of calcium in milk solids and in calcium gluconate was followed throughout forty-three 4-day periods.

For equilibrium 9.2 mg. of calcium per kilogram of body weight per day were required, about three-fourths of which were supplied either in milk solids or in calcium gluconate. The calcium in both of these forms was equally well utilized, but only to the extent of 20%.

Grateful acknowledgment is made to Dr. T. S. Hamilton for advice concerning the chemical methods used in this investigation.

LITERATURE CITED

ADOLPH, W. H., AND S. C. CHEN 1932 The utilization of calcium in soy bean diets. J. Nutrition, vol. 5, pp. 379–385.

KRAMER, M. M., E. LATZKE AND M. M. SHAW 1928 A comparison of raw, pasteurized, evaporated and dried milks as sources of calcium and phosphorus for the human subject. J. Biol. Chem., vol. 79, pp. 283–295.

KRAMER, M. M., M. T. POTTER AND I. GILLUM 1931 Utilization by normal adult subjects of the calcium and phosphorus in raw milk and in ice cream. J. Nutrition, vol. 4, pp. 105–114.

MALLON, M. G., L. M. JOHNSON AND C. R. DARBY 1932 A study of the calcium retention on a diet containing American cheddar cheese. J. Nutrition, vol. 5, pp. 121–126.

——— 1933 The calcium retention on a diet containing leaf lettuce. J. Nutrition, vol. 6, pp. 303–311.

MALLON, M. G., R. JORDAN AND L. M. JOHNSON 1930 A note on the calcium retention on a high and low fat diet. J. Biol. Chem., vol. 88, pp. 163–167.

MITCHELL, H. H., AND E. G. CURZON 1939 The dietary requirement of calcium and its significance. To be published as one of a series of monographs on nutrition edited by E. F. Terroine. Paris.

ROSE, M. S. 1920 Experiments on the utilization of the calcium of carrots by man. J. Biol. Chem., vol. 41, pp. 349–355.

ROSE, M. S., AND G. MACLEOD 1923 Experiments on the utilization of the calcium of almonds by man. J. Biol. Chem., vol. 57, pp. 305–315.

SHERMAN, H. C., AND E. HAWLEY 1922 Calcium and phosphorus metabolism in childhood. J. Biol. Chem., vol. 53, pp. 375–399.

THE LACTATION-PROMOTING EFFECT OF l-CYSTINE WHEN FED WITH ALFALFA PROTEINS[1]

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ONE FIGURE

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In a previous paper (Haag, '31) it was shown that the growth-promoting properties of alfalfa crude protein are improved by additions of l-cystine. This finding has since been confirmed and extended by Kellermann ('35). In the light of recent work dealing with the interchangeability of cystine and methionine, it appears that these and other cystine studies may need re-interpretation to the extent that cystine functions in supplementing a deficiency of methionine. More recently, Haag and Wright ('38) have reported preliminary studies in which the growth of nursing rats, as a measure of lactation, indicates that additions of l-cystine enhance the lactation-promoting properties of alfalfa crude protein. These studies are in harmony with much of our information dealing with the nutritive deficiencies of certain legume proteins and with recent (Daggs and Tomboulian, '35; Daggs and Lidfeldt, '38) work which has been interpreted as indicating that cystine may be looked upon as a dietary lactation stimulant. It is our purpose in this paper to present in detail some of our recent studies dealing with the influence of l-cystine on lactation in rats fed on rations in which the principal source of nitrogen is obtained from commercial alfalfa leaf meal.

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[2] The data reported in this paper are to be presented by L. D. Wright in partial fulfillment of the requirements for the degree of doctor of philosophy, Oregon State College.

PROCEDURE

The general procedure has been to subject rats in advanced stages of pregnancy to a preliminary feeding period on a ration (no. 113) containing 50% of alfalfa leaf meal. As soon as young are born, the experimental ration (no. 114 or 115) is substituted for the preliminary ration. Daily records are kept of the weights of the female and of the litter of young. Not later than the second or third day the litter is reduced to six young. Feed intake records are kept on a weekly basis. Data are given for all females which completed a lactation period of 21 days with six young.

TABLE 1

Composition of rations used

INGREDIENTS	RATION NO.		
	113	114	115
Alfalfa leaf meal (2.99% N)	50.0	50.0	50.0
Starch	10.7	20.0	19.6
Sucrose	5.0	5.0	5.0
Hydrogenated fat	19.0	19.0	19.0
Cod liver oil	1.0	1.0	1.0
Yeast	3.0	3.0	3.0
$NaH_2PO_4 \cdot H_2O$	1.5	1.5	1.5
NaCl	0.5	0.5	0.5
Casein	9.0	...	...
l-cystine	0.3	...	0.4

RATIONS

The rations are of the same general type as those previously used (Haag, '31) in our growth studies. The composition of the rations used is given in table 1. The apparent digestibility of the dry matter in rations 114 and 115 is 72%; that of the crude protein 60%. The alfalfa leaf meal contained 0.37% total and 0.18% organic sulphur.

EXPERIMENTAL RESULTS

The results obtained are in part tabulated in table 2. These results include weights of the litters of six young for 0, 3, 17 and 21 days following birth, the day of birth being designated as the '0' day. The use of day 3 as a starting point permits postponing reducing the litter to six young and the use of day 17 is justified on the basis that it approximately marks the end of the period when growth may be attributed to an exclusive milk diet.

TABLE 2

Live weight and feed consumption data on lactating females and weight of young

RAT NO.	DAILY FEED	WEIGHT OF FEMALES				WEIGHT OF SIX YOUNG			
		Days				Days			
		0	3	17	21	0	3	17	21
					Ration 114				
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
2057	18.3	229	217	162	157	32	39	81	85
2058	13.7	240	232	168	155	—	39	82	85
2060	17.6	297	280	198	184	36	50	84	96
2070	14.3	250	245	169	159	32	42	80	82
2161	12.2	332	309	220	208	34	43	86	88
2162	17.4	263	263	177	173	—	39	92	94
2300	16.9	265	258	187	183	36	45	96	110
2301	19.5	240	222	170	160	35	43	93	103
2303	16.2	220	215	160	154	29	37	75	79
Ave.	16.2	260	249	179	170	33	42	85	91
					Ration 115				
1864	23.1	320	312	267	248	37	55	142	171
2057	19.0	279	255	220	209	33	50	118	130
2058	17.9	245	235	206	198	35	44	112	125
2060	27.0	271	267	238	226	—	48	120	135
2061	20.7	285	284	—	239	35	55	—	157
2063	24.5	278	272	256	234	31	45	132	147
2145	17.4	282	270	231	220	35	48	126	147
2161	23.8	282	290	262	254	—	47	141	152
Ave.	21.7	280	273	240	229	34	49	127	146

In figure 1 are found the live weight curves for the litters of young rats. These curves are, with a few minor exceptions, plotted on a daily basis.

DISCUSSION

The graphic presentation in figure 1 emphasizes the striking character of the results obtained. The response to cystine feeding is marked and the uniformity of response to the respective rations is equally marked. The significance of the response to cystine feeding, as reflected in the growth of the nursing young is enhanced by the effect of cystine in retarding the loss of weight by the lactating female. It will be noted that the cystine supplement usually increased the food intake, approximately doubled the rate of gain of the nursing young, and reduced the rate of body weight loss of the lactating

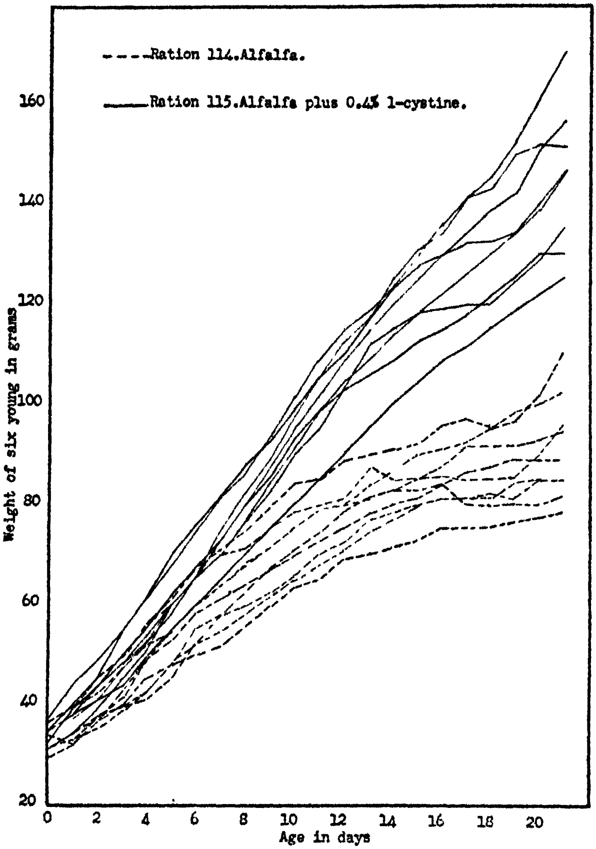

Fig. 1 Growth curves for litters of young.

females approximately 50%. The average 'lactation index' values, as suggested by Daggs ('35), were 382 and 586, respectively, for rations 114 and 115.

In this group of experiments there was no predetermined plan for controlling the total food intake. During the first week of lactation the average food intakes on rations 114 and 115 were not significantly different. During the second and third weeks, however, the cystine supplemented ration was consumed far more freely.

This report would be incomplete without some reference to the ultimate significance of our results. Their application to practical alfalfa feeding problems is in part conditioned by the level (approximately 9%) of protein studied as compared to the 12 to 15% level commonly found in alfalfa hay. A most useful implication lies in the direction of developing more precise methods for evaluating proteins for their lactation-promoting properties.

The levels of alfalfa proteins at which cystine responses are effective, the response to various levels of cystine, the development of controlled-feeding technics analogous to the paired-feeding method, the response to other amino-acids, the determination of supplementary effects, and finally the determination as to whether the amino acid requirements for growth and lactation are of the same order, are questions which are now being investigated.

SUMMARY

The lactation-promoting properties of rations containing approximately 9% of alfalfa crude protein are markedly enhanced by 0.4% additions of l-cystine.

LITERATURE CITED

DAGGS, R. G. 1935 Studies on lactation. II. Technic for studying lactation in rats. J. Nutrition, vol. 9, p. 575.

DAGGS, R. G., AND VIOLA S. M. LIDFELDT 1938 The effect of the sulphydryl compounds on milk production. J. Nutrition, vol. 15, p. 211.

DAGGS, R. G., AND R. L. TOMBOULIAN 1935 Studies on lactation. III. Effect of various dietary principles on lactation in rats. J. Nutrition, vol. 9, p. 581.

HAAG, J. R. 1931 The physiological effect of rations restricted principally or solely to the alfalfa plant. II. Cystine as a limiting factor in the nutritive value of alfalfa proteins. J. Nutrition, vol. 4, p. 363.

HAAG, J. R., AND L. D. WRIGHT 1938 The nutritive value of alfalfa proteins as measured by lactation in rats. Proceedings 24th Annual Meeting, Western Division, American Dairy Science Association.

KELLERMANN, J. H. 1935 Further observations on the cystine deficiency of lucerne proteins and the effect of heat and incubation upon their growth-promoting value. Onderstepoort J. Vet. Sci., vol. 4, p. 437.

THE DISTRIBUTION OF VITAMIN B_1 (THIAMIN) IN MEAT AND MEAT PRODUCTS[1]

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The value of meat and meat products as sources of protein, energy and minerals in the human dietary is generally recognized, but much less attention has been given to the vitamin content of these substances. With the increasing knowledge of the water soluble vitamins associated in the so-called B complex, it becomes more and more evident that meat and meat products cannot be ignored as important sources of many of these factors. The separation of the old factors into several vitamins and the recognition of new ones necessitates the reappraisal of many of the values now found in the literature.

The available information on the B complex is far from complete but at least six members, namely, thiamin, riboflavin, nicotinic acid, vitamin B_6, chick antidermatitis factor and factor W may be recognized as separate entities. There is need for much improvement in the methods used for the biological assay of these vitamins, but through the use of rats, chicks and dogs we have been able to make rather extensive studies on the distribution of these factors in meats. The studies on thiamin are reported in this paper.

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REVIEW OF THE LITERATURE

The investigations on the vitamin B content of meats made before 1923 were reviewed by Hoagland ('23 a). He ('23 b, '29) went on to study this question further by means of the pigeon protective method and found thereby a high content of this vitamin in pork muscle. Umishio ('30) determined the minimum amount of an alcoholic extract of raw and canned meat required for normal growth of rats on a B deficient ration. Roscoe ('31) studied this problem by means of the rat growth method. Christensen and co-workers ('36) studied the influence of cooking and canning on the vitamin B_1 content of lean beef and pork. Kellogg ('36) by means of a rat growth method found vitamin B_1 to be fairly evenly distributed in lamb tissues and organs. Plimmer et al. ('33) used pigeons in determining the antineuritic properties of meat. Baker and Wright ('35) used the bradycardia method on rats in their assays. Elvehjem et al. ('35) used chicks on an autoclaved ration in their studies. Recently Williams and Spies ('38) have summarized the vitamin B_1 content of animal tissues as determined by the various methods. The wide range of values for this vitamin in the same tissue gives ample proof for the necessity of further studies on vitamin B_1.

EXPERIMENTAL

The vitamin B_1 content of the tissues was determined by means of the rat growth method. The basal diet used was ration 112 described by Arnold and Elvehjem ('38 a) which has been shown to be complete in all respects except vitamin B_1. This ration was prepared as described by the above workers.

Each series was composed of about forty rats weighing between 35 and 45 gm. which were placed in individual screen bottom cages. All rats were kept on the basal ration for 1 week. Then a group of two rats, a male and a female, was placed on the supplemented basal ration containing a definite per cent of the tissue. In each series one group, which served as the negative control, was kept on the basal ration, while the standard group received 80 micrograms of crystalline vitamin B_1 per 100 gm. of ration. The dried tissue was added to the basal ration at various levels in order to find the level which produced a gain in weight comparable to that of the standard group in that particular series.[2] All assays were made over a 6-week period during which time the rats were weighed weekly. The increases in body weight of the test and standard groups were compared and the vitamin B_1 potency calculated by means of a simple proportion.

The tissues used in these studies were from animals which had been slaughtered a day or so before shipment to the laboratory. All of the tissues were from animals which were graded at the time of slaughter as excellent.[3] As soon as the tissues arrived at our laboratory they were placed in a cold room after which they were prepared for drying.

Our first efforts were directed toward the development of a method for drying the tissues with as little destruction of vitamin B_1 as possible. Preliminary work indicated that tissues dried under vacuum at temperatures above 70°C. showed appreciable destruction of vitamin B_1. These tissues were prepared by separating the meat from as much fat and tendon as possible. The lean meat was then finely ground and spread out in thin layers (1 to 3 mm.) on pans over which a current of warm air (45 to 60°C.) was passed. The time required for drying has usually been 6 to 10 hours. The chemical analyses of these tissues as well as all other tissues assayed are given in table 1.

To determine whether any vitamin B_1 was destroyed during our drying process, both the fresh and the dried meat from the same animal were assayed simultaneously. The meat which was to be fed in the fresh condition was kept frozen

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[2] The crystalline vitamin B_1 was accurately weighed out and made up to a definite dilution in N/10 HCl. This standard was added to a small part of the basal ration, dried in a vacuum desiccator and then mixed with a sufficient amount of the basal ration to produce a concentration of 80 micrograms of vitamin B_1 per 100 gm. of ration.

[3] We wish to thank Swift and Company, Armour and Company and Wilson and Company for furnishing samples of fresh tissues.

TABLE 1

Composition of animal tissues used in these studies

MEAT	MEAT PROCESS	MEAT SAMPLE	MOISTURE IN ORIGINAL SAMPLE	FAT IN DRIED SAMPLE	PROTEIN IN DRIED SAMPLE
			%	%	%
Beef brain	Dried	57	79.1	48.6	46.7
Beef heart	Dried	53	77.5	12.6	78.2
Beef heart	Stewed	54		11.3	81.4
Beef kidney	Dried	49	78.9	13.1	73.8
Beef kidney	Stewed	50		13.2	72.4
Beef liver	Dried	58	67.6	18.3	64.2
Beef lung	Dried	63	77.9	10.2	75.9
Beef pancreas	Dried	64	67.9	41.2	56.4
Beef round	Dried	40		16.6	75.6
Beef round	Broiled	41		23.1	73.0
Beef round	Fried	42		16.6	77.0
Beef round	Roasted	43		15.2	78.6
Beef spleen	Dried	59	76.8	12.8	77.4
Beef tongue	Dried	67	68.4	41.7	54.5
Veal hindquarter	Dried	44		15.8	79.2
Veal hindquarter	Fried	45		14.8	77.5
Veal hindquarter	Roasted	46		4.2	83.0
Veal liver	Dried	70		20.2	65.2
Veal, leg of	Dried	56	76.4	12.4	81.5
Lamb liver	Dried	61	65.5	23.5	57.3
Lamb, leg of	Dried	35		22.2	72.6
Lamb, leg of	Dried	55	72.3	21.3	74.1
Pork ham	Dried	32	72.5	19.0	76.2
Pork ham	Dried	51	74.5	21.2	74.2
Pork ham	Fried	52		20.4	76.5
Smoked ham	Dried	47	67.5	14.4	63.1
Smoked ham	Fried	48		16.0	62.6
Canned ham	Dried	72	68.7	10.8	67.6
Pork heart	Dried	71		14.4	70.4
Pork kidney	Dried	62	78.4	18.6	72.8
Pork liver	Dried	33	70.5	13.8	55.8
Pork loin	Dried	34	71.5	24.3	57.4
Pork loin	Dried	36		31.0	54.5
Pork loin	Baked	37		34.4	59.3
Pork loin	Fried	38		28.4	62.8
Pork loin	Roasted	39		30.8	61.3
Poultry, dark	Dried	66	74.2	18.8	75.2
Poultry, dark	Dried	69	76.4	12.2	80.4
Poultry, light	Dried	65	72.8	11.6	84.4
Poultry, light	Dried	68	75.4	5.4	83.9

in the cooler till needed. Daily food consumption records were kept on all rats receiving various levels of pork ham, pork loin and pork liver. In another series the rats received the fresh tissues in daily supplements equivalent to the dry tissues consumed by the rats in the corresponding group in the first series. The rats in the second series had free access at all times to the basal ration. These results (table 2) show that the dried pork ham (sample 33) contained 11 I.U.[4] of vitamin B_1, which agrees well with 10 I.U. per gram (on the dry weight basis) for the fresh tissue. The values of the vitamin B_1 content of the fresh and dried pork loin and liver are within experimental error. This agreement warrants the conclusion that very little if any vitamin B_1 is destroyed by our method of drying.

We therefore proceeded to collect, dry and assay a variety of meats and meat products. The results show an exceedingly high content of vitamin B_1 in pork muscle. The pork loin (sample 34) used in the above studies contained 13 I.U. per gram. It was from an animal killed in September. Another sample of pork loin (sample 36) from an animal slaughtered in November contained 20 I.U. of vitamin B_1 per gram. A similar variation was noted in some samples of pork ham. One sample secured in September (sample 33) from the same animal as the first pork loin (sample 34) contained 11 I.U. per gram of dried material. A second sample of ham (sample 52) was secured in January of the following year from a different source. This sample had 20 I.U. of vitamin B_1 per gram of dried material.

The values for the vitamin B_1 content of pork muscle are considerably higher than those of other meats. Beef round contained 3 I.U. of vitamin B_1 per gram and two samples of leg of lamb contained 4 I.U. of vitamin B_1 per gram. One sample of leg of veal secured in December contained 4.5 I.U. of vitamin B_1 per gram whereas a later sample secured in March contained 1.6 I.U. per gram. Excluding

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[4] The conversion factor 1 I.U. = 3 micrograms of crystalline vitamin B_1 was used (Arnold and Elvehjem, '38 b).

TABLE 2

The vitamin B_1 content of meat and meat products

MEAT	MEAT SAMPLE	MEAT PROCESS	EXPERIMENT NO.	VITAMIN B_1 CONTENT	
				γ per gram[1]	I.U. per gram[1]
Beef brain	57	Dried	1883	8	2.7
Beef heart	53	Dried	1854	30	10
Beef heart	54	Stewed	1857	13	4.5
Beef kidney	49	Dried	1894	15	5
Beef kidney	50	Stewed	1895	9	3
Beef liver	58	Dried	2827	12	4
Beef lung	63	Dried	2810	9	3
Beef pancreas	64	Dried	2811	10	3.3
Beef round	40	Dried	1826	9	3
Beef round	41	Broiled	1858	4.5	1.5
Beef round	43	Roasted	1872	4[2]	1.3[2]
Beef round	42	Fried	1832	9	3
Beef spleen	59	Dried	1887	7.0	2.5
Beef tongue	67	Dried	2824	9	3
Lamb liver	61	Dried	2826	12	4
Lamb, leg of	35	Dried	884	12	4
Lamb, leg of	55	Dried	1876	12	4
Veal hindquarter	44	Dried	1834	13.5	4.5
Veal hindquarter	56	Dried	2844	5.2	1.6
Veal hindquarter	45	Fried	1836	7.5	2.5
Veal hindquarter	46	Roasted	1874	6	2
Veal liver	70	Dried	2845	5.2[2]	1.7[2]
Pork ham	32	Dried	1805	33	11
Pork ham	32	Fresh	1813	30	10
Pork ham	51	Dried	1850	60	20
Pork ham	52	Fried	1852	60	20
Smoked ham	47	Dried	1843	33	11
Smoked ham	48	Fried	1845	29.0	9.5
Canned ham	72	Dried	2846	26	8.6
Pork heart	71	Dried	2848	25.5	8
Pork kidney	62	Dried	1893	24	8
Pork liver	33	Dried	875	16	5.3
Pork liver	33	Fresh	876	15	5
Pork loin	34	Dried	1807	39	13
Pork loin	34	Fresh	1815	39	13
Pork loin	36	Dried	897	60	20
Pork loin	37	Baked	891	30	10
Pork loin	38	Fried	893	39	13
Pork loin	39	Roasted	895	30	10
Poultry, dark	66	Dried	2816	9	3
Poultry, dark	69	Dried	2837	7.6	2.5
Poultry, light	65	Dried	2839	<5.5	<1.8
Poultry, light	68	Dried	2836	3[2]	1[2]

[1] On the dry weight basis.

[2] Calculated—the level of tissue could not be fed high enough for optimal growth.

veal, the livers of the other animals were remarkably constant in their vitamin B_1 potency in spite of rather wide fluctuations in the content of this vitamin in their muscles. The livers from beef, hog and lamb contained approximately the same amount of vitamin B_1, 4 to 5.3 I.U. per gram of dried material. In contradistinction to these values was that of veal liver which contained only 1.7 I.U. of vitamin B_1 per gram. This sample was a composite of three calves which were slaughtered in July.

Beef heart was a very good source of vitamin B_1. Our sample, which was a composite from eight different animals, contained 10 I.U. of vitamin B_1 per gram of dried material. Pork heart contained slightly less than this or 8 I.U. of vitamin B_1 per gram. There were 8 I.U. in each gram of pork kidney whereas beef kidney had 5 I.U. per gram. In both of these cases the kidney was richer in vitamin B_1 than the liver of the same animal. Beef lung and beef tongue both contained 3 I.U. of vitamin B_1 per gram, making them as potent as the muscles from these animals. In the case of beef brain, the gray matter was separated from the white and only the white matter was used. According to our assays there were 2.7 I.U. of vitamin B_1 per gram of dried brain. Our values for beef kidney, beef lung and beef spleen agree well with those reported by Arnold and Elvehjem ('38 c) who used the chick growth method of assay. They found these tissues to contain 5, 2 and 2 I.U. per gram of dried tissue respectively.

The first samples of poultry which we assayed were from two cocks which were nearly 3 years old. The dark meat on the lower legs and abdomen and the light meat on the breast and wings were prepared separately. The second samples of poultry were from fifteen pullets about 5 months old. In both cases the assays for vitamin B_1 indicated a higher content of the vitamin in the dark meat than in the light. The dark meat from the older birds contained 3 I.U. of vitamin B_1 per gram of dried material while that from the younger animals contained 2.5 I.U. per gram. The light meat in the older birds contained less than 1.8 I.U. of vitamin B_1 per gram and that from the younger animals contained about 1 I.U. per gram.

The samples which were used for cooking were cuts of meat such as are sold commercially. They were prepared according to the directions which have been described by Child ('38). In each case a part of the uncooked sample was dried according to our method and this was used in determining the original vitamin B_1 content of the tissue. The cooked and uncooked meats were assayed simultaneously for vitamin B_1 by the rat growth method. From the difference in the vitamin B_1 content of these samples the amount of destruction of the vitamin was calculated (table 3). These results indicate that the stability of vitamin B_1 in meats during cooking depended to a certain extent upon the method of cooking. Frying apparently produced the least change in the antineuritic vitamin as shown by the almost complete preservation of the vitamin in fried beef round and fried pork ham. When smoked ham was fried some 10% of the

TABLE 3

Stability of vitamin B_1 during household cooking processes

PROCESS	MEAT	MEAT SAMPLE	TIME OF PROCESSING	DESTRUCTION OF VITAMIN B_1
Frying	Beef round	42	20 minutes	0
	Veal hindquarter	45	20 minutes	45%
	Pork ham	52	15 minutes	0
	Smoked ham	48	15 minutes	10%
	Pork loin	38	Medium well done	35%
Roasting	Beef round	43	2.5 hours	61%
	Veal hindquarter	45	2 hours	58%
	Pork loin	39	1.5 hours	50%
Broiling	Beef round	41	20 minutes	50%
Baking	Pork loin	37	1 hour	50%
Stewing	Beef heart	54	1 hour	55%
	Beef kidney	50	45 minutes	40%

vitamin was destroyed. This was increased to 35% in the case of pork loin and 45% in veal chops.

Roasting seemed to have a greater destructive action on the vitamin B_1 in the three samples tested, for this method of cooking inactivated from 50 to 60% of the vitamin. The beef round, veal hindquarter and pork loin showed a greater preservation of the vitamin with a decrease in the time of roasting. The broiled beef round and baked pork loin both showed a 50% destruction of the vitamin B_1 content after cooking. The stewed beef heart and stewed beef kidney showed a stability of the vitamin similar to that of the broiled meat. The rather marked destruction of the vitamin produced by roasting, broiling, baking and stewing is possibly associated with the long time and high temperature used in cooking.

Since several workers, Evans and Lepkovsky ('29), Salmon and Goodman ('37), Stirn and Arnold ('38) have shown that fat decreases the vitamin B_1 requirement of rats, we determined whether the fat content of these tissues had any influence on our biological assays. A series of rats was fed various levels of coconut oil and suboptimal amounts of vitamin B_1 as supplements to our basal ration. By this means it was found that the rats receiving added fat in their ration up to levels of 20% grew no better than those getting only the corresponding amount of vitamin B_1. The level of tissue required for an assay has usually been less than 10%. This definitely indicates that the fat in the tissues assayed had no influence on the result of these experiments.

DISCUSSION

In any study on the quantitative distribution of specific nutrients, the figures have little significance unless they are related to human requirements. This paper indicates that our meat consumption is able to supply a considerable fraction of our daily vitamin B_1 requirement.

Investigations on the vitamin B_1 content of our daily foods have shown that most of these substances contain from 0.5

to 2.0 I.U. of vitamin B_1 per gram. Our work shows that meats are above the average in this respect and compare favorably with many foods which are ordinarily considered potent sources of this vitamin. For instance, Baker and Wright ('35) found different samples of whole wheat to contain from 2.3 to 3.4 I.U. of vitamin B_1 per gram while our values for meats show them to contain 2 or more I.U. per gram. Even after cooking, meats are still within the above range of vitamin potency.

Although there are no accurate figures indicating the amount of meat used in the average American diet, the figures of Stiebling and Ward ('33) indicate that the typical diet contains about 7% of meat. The average daily food consumption would have to approximate 700 gm. in order to supply a daily energy requirement of about 2700 Calories. Since about 7% of the daily food is meat, then this constituent will supply not less than 150 I.U. of vitamin B_1 per day (on the basis that meat contains 3 I.U. of vitamin B_1 per gram). A confirmation of this value is obtained by calculating the amount of vitamin B_1 in the yearly meat consumption of city wage earners. According to calculations based on the most recent statistics of this sort (Stiebling, '38), an average person secures over 200 I.U. of vitamin B_1 each day from his meat intake.

The daily human requirement for vitamin B_1 has been placed at 200 to 500 I.U. (Williams and Spies, '38). Therefore, even though meats are used in the diet to the extent of only 7%, they are capable of supplying one-third of the human vitamin B_1 needs. When meat such as pork is used, the proportion of the day's requirement thus supplied is correspondingly increased.

A pork chop, even when fried, may supply the total daily requirement of this vitamin. An ordinary pork chop contains approximately 25 gm. of dried meat. Our sample of fried pork loin (sample 38) contained 13 I.U. of vitamin B_1 per gram which means that there may be 325 I.U. of vitamin B_1 in a pork chop.

SUMMARY

1. A biological method of assaying various materials for their vitamin B_1 content has been described. This is based on a comparison of the growth of rats on a basal ration containing the material to be assayed with the growth of rats on the basal ration with added amounts of crystalline vitamin B_1.

2. A simple method of drying moist materials, which minimizes destruction of vitamin B_1, has also been described.

3. By our method of assay a large number of meats and meat products have been tested for their vitamin B_1 content. A study has also been made on the stability of the vitamin under various types of household cooking. Here it was found that there was slight destruction of the vitamin during frying, but with roasting, broiling or stewing, the destruction of the vitamin approached 50%.

4. A study of the influence of fats on the growth of the rats on our basal ration has been made. Our work showed that levels of fat up to 20% had no influence on the validity of our assays.

LITERATURE CITED

ARNOLD, A., AND C. A. ELVEHJEM 1938 a Studies on the vitamin B_1 requirements of growing rats. J. Nutrition, vol. 15, p. 429.

——— 1938 b Studies on the vitamin B_1 requirements of growing chicks. J. Nutrition, vol. 15, p. 403.

——— 1938 c Stability of vitamin B_1 of vacuum-dried animal tissues during storage. Food Research, vol. 3, p. 367.

BAKER, A. Z., AND M. D. WRIGHT 1935 The vitamin B_1 content of foods. Biochem. J., vol. 29, p. 1802.

CHILD, A. M. 1938 Recent developments in meat cookery research. J. Am. Diet. Assoc., vol. 14, p. 623.

CHRISTENSEN, F. W., E. LATZKE AND T. H. HOPPER 1936 The influence of cooking and canning on the vitamin B and G content of lean beef and pork. J. Agr. Research, vol. 53, p. 415.

ELVEHJEM, C. A., W. C. SHERMAN AND A. ARNOLD 1935 The vitamin B(B_1) content of animal tissues. J. Biol. Chem., vol. 109, Proceedings XXIX.

EVANS, H. M., AND S. LEPKOVSKY 1929 Sparing action of fat on the antineuritic vitamin. J. Biol. Chem., vol. 83, p. 269.

HOAGLAND, R. 1923 a Vitamin B in the edible tissues of ox, sheep and hog. U. S. Dept. Agri., Bull. no. 1138.

——— 1923 b Antineuritic value of hog muscle Am. J. Physiol., vol. 67, p. 300.

——— 1929 Antineuritic and water soluble B vitamins in beef and pork. J. Agr. Research, vol. 38, p. 431.

KELLOGG, M. 1936 Vitamin B in lamb tissues and organs. S. Dakota Agr. Exp. Sta. Bull. no. 306.

PLIMMMER, R. H. A., W. H. RAYMOND AND J. LOWNDES 1933 Comparative vitamin B values of animal foodstuffs. Biochem. J., vol. 27, p. 58.

ROSCOE, M. H. 1931 The distribution of the vitamin B complex. Biochem. J., vol. 25, p. 2050.

SALMON, W. D., AND J. G. GOODMAN 1937 Alleviation of vitamin B deficiency in the rat by certain natural fats and synthetic esters. J. Nutrition, vol. 13, p. 477.

STIEBLING, H. K. 1938 Personal communication.

STIEBLING, H. K., AND M. M. WARD 1933 Diets at four levels of nutritive content and cost. U. S. Dept. Agri. circular 296.

STIRN, F. E., AND A. ARNOLD 1938 The relation of dietary fat to the vitamin B_1 requirement of growing rats. J. Biol. Chem., vol. 123, p. cxvii.

UMISHIO, Y. 1930 Vitamin B content of raw and canned beef. Imp. Zootech. Exp. Sta. (Japan) Bull. 25 (Chem. abst. 25), 2207 (1931).

WILLIAMS, R. R., AND T. D. SPIES 1938 Vitamin B_1 and its use in medicine. P. 236. Macmillan and Co., New York, N. Y.

THE REQUIREMENTS OF THE DOG AND THE RAT FOR NICOTINIC ACID

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THREE FIGURES

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I. THE MINIMUM AMOUNT REQUIRED BY THE DOG

The work of Elvehjem et al. ('37, '38) has made it evident that nicotinic acid and nicotinic acid amide are effective in curing the deficiency disease known as canine blacktongue. Other workers, Street and Cowgill ('37), Smith et al. ('38), have confirmed this observation. Fouts et al. ('37) have shown that human pellagra may be cured by feeding 500 mg. of nicotinic acid per day. Spies et al. ('37) also found that the mucous membrane lesions of pellagrins could be cured by feeding nicotinic acid. Chick et al. ('38) fed 60 mg. nicotinic acid per day to swine on pellagra producing diet and found that it induced a normal growth rate and maintained the animals in good condition.

The minimum need for nicotinic acid has not, however, been determined for any species. Elvehjem et al. ('37, '38) were able to cure acute blacktongue with a single dose of 25 or 30 mg. of nicotinic acid feeding 100 mg. per week subsequently prevented the reappearance of symptoms. Street and Cowgill ('37) obtained good growth with dogs by feeding 25 or 50 mg. per dog per day, while Smith et al. ('38) obtained growth with 1.0 mg. nicotinic acid per day per kilogram body weight. The effect of lower doses has not been reported except for a recent publication by Sebrell et al. ('38) which is referred to later.

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The following experiments were, therefore, undertaken with the object of determining the minimum daily requirements of the dog for nicotinic acid.

EXPERIMENTAL

The ainmals used in these experiments were adult dogs of mixed breed, weighing 5 to 10 kg. The diet consisted of:

	%
Yellow cornmeal	74.0
Pea meal	8.0
Casein (alcohol extracted)	9.0
Cottonseed oil	6.0
Sodium chloride	0.5
Calcium carbonate	1.0
Calcium phosphate	1.0
Iron oxide	0.04

The diet was cooked fresh each day by adding water to it and steaming in a steam cooker for 2 hours; 1% of cod liver oil was mixed in the diet after cooking. Synthetic thiamin and pure crystalline riboflavin were fed at levels of 0.1 mg. and 0.5 mg. per dog per day respectively.

A combination of the curative and protective methods have been used for assaying the minimum amount of nicotinic acid necessary. In the earliest experiments graded daily doses of nicotinic acid were fed to the animals after they had been on the deficient diet for several weeks, but had no definite symptoms of blacktongue. It was found that the lowest amount tried 2.5 mg. per day produced a rapid increase in weight and complete protection (fig. 1).

For assaying smaller amounts a slightly different method was used. The animals were first run out of nicotinic acid until they showed definite symptoms of blacktongue. The symptoms were then cured by injecting 10 mg. of nicotinic acid on 3 successive days. The amount of nicotinic acid was then reduced to the lower levels of 0.25, 0.5 and 1.0 mg. per day which was given by mouth. Blacktongue symptoms again developed when 0.25 and 0.5 mg. were fed; with 1.0 mg., however, complete protection and slow increase in weight resulted over a period of 12 weeks. These results are shown in figure 1.

In later experiments attempts were made to cure animals with blacktongue by giving 1.0 mg. of nicotinic acid per day, without first giving the 30 mg. dose. When the symptoms were well marked this method always failed to bring about a cure. If, however, the 1.0 mg. dose was started before severe symptoms had developed the animals would be protected and commence gaining in weight slowly. These results are shown in figure 1 (animals 5 and 11). It was found necessary to feed 15 gm. of yeast per day to the positive controls in order to get a steady increase in weight. The minimum protective dose of yeast may, however, be smaller than this.

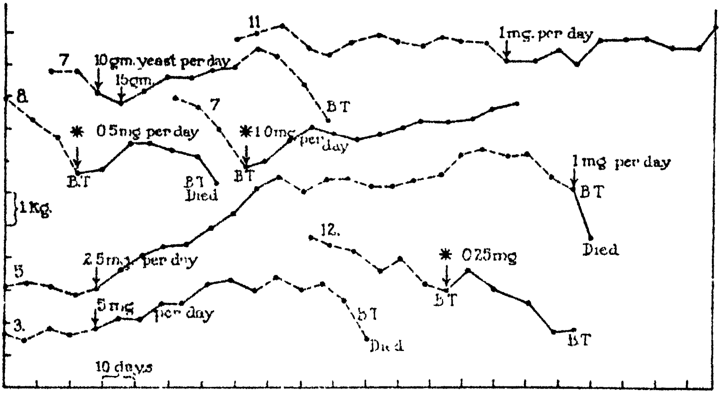

Fig. 1 Growth curves of dogs fed Goldberger diet. Broken lines indicate that the deficient diet alone was being fed. Continuous lines indicate that a supplement of either yeast or nicotinic acid was given. * = 30 mg. of nicotinic acid was injected in three successive daily doses of 10 mg.

DISCUSSION

It is evident from these experiments that the minimum dose of nicotinic acid necessary to protect the dog against blacktongue is about 1.0 mg. per day. When the results are worked out on the basis of milligrams of nicotinic acid fed per kilogram of body weight it is found that 0.25 mg. per kilogram gave rapid increase in weight and complete protection; 0.13 mg. per kilogram gave complete protection and a slow increase in weight; while 0.084 and 0.027 mg. per kilogram gave no protection.

Considerable variation in the time which dogs take to develop blacktongue after being changed from an adequate diet to the deficient one has been noted. Thus dog no. 3 which had been having 5 mg. of nicotinic acid developed blacktongue in 5 weeks after the dose had been stopped. While no. 5 which had been receiving 2.5 mg. per day took 13 weeks to develop symptoms. When dogs have just been cured of blacktongue by a single large dose of nicotinic acid, the symptoms quickly appear again unless the dose is repeated. This would indicate that the general nutritive condition of the animal plays a part in determining the time for the onset of the disease, rather than the actual amount of nicotinic acid which the dogs have been receiving previous to giving the deficient diet.

These results are substantially in agreement with those reported by Sebrell et al. ('38). These authors found that when a semi-weekly dose of 1 mg. nicotinic acid, equivalent to 0.28 mg. per day was given, blacktongue developed. When the equivalent of 0.85 mg. per day was fed, some symptoms of incipient blacktongue appeared, but with higher doses complete protection was maintained. They also found that the curative method gave highly irregular results. The amount of nicotinic acid necessary to cause the symptoms of blacktongue to subside appeared to be between 20 and 60 mg. The duration of the cure, however, was not strictly proportional to the dose given and large variations were encountered.

It is interesting to compare the minimum protective dose of nicotinic acid for the dog with that of yeast found by Goldberger et al. ('28). These workers found that when 1.8% of yeast was mixed with the blacktongue producing diet, almost complete protection was observed over a period of more than 300 days. This percentage appears to be roughly equivalent to 4 gm. of yeast per day. In curative experiments they found it necessary to feed 20 gm. of yeast per day to a 10 kg. dog to obtain a cure. Afterward 10 gm. of yeast per dog were required to prevent a recurrence of symptoms. This is approximately the same amount as was used here for the positive controls. Thus it would appear that 1.0 mg. of nicotinic acid is contained in 4 to 10 gm. of yeast.

Goldberger and Tanner ('25) found that 50 gm. of yeast per day was sufficient to cure pellagra encountered among colored females at the Georgia State Sanatorium. This would suggest, therefore, that 5 to 12 mg. of nicotinic acid should be sufficient to cure human pellagra. This amount is much smaller than that used by Fouts et al. ('37) and Spies et al. ('37) who fed as much as 500 to 1000 mg. per dag.

A recent observation by Schmidt and Sydenstricker ('38) has shown that when 90 gm. of yeast per day were fed to a group of pellagrins, a marked improvement in their condition occurred. When 100 mg. of nicotinic acid were given twice weekly to another group some improvement was noted at first, but it was not maintained. At the end of a 6-week period their condition was little better than it was before treatment.

These workers suggest that 90 gm. of yeast are equivalent to 15 mg. of nicotinic acid, which is in agreement with the calculation made here. From these observations it would seem that yeast is more effective in the treatment of pellagra than is the equivalent amount of nicotinic acid calculated on the basis of experiments with dogs.

It must be pointed out, however, that in Schmidt and Sydenstricker's experiments the diet of the pellagrins was not strictly controlled and may have been deficient in other factors such as riboflavin. These factors would be supplied by yeast and may help in bringing about a complete recovery.

II. IS NICOTINIC ACID AN ESSENTIAL SUBSTANCE FOR THE RAT?

It is still uncertain whether nicotinic acid is an essential substance for the rat or not. Rhodes and Miller ('35); Birch, Gyorgy and Harris ('35); Birch, Chick and Martin ('37) found that rats fed on pellagra producing diets were able to thrive, whereas dogs or swine reared on a similar diet rapidly succumbed. Since it has been shown that nicotinic acid is the substance missing from the pellagra producing diet, it would be presumed that rats do not need this substance. Other workers, however, have presented evidence which indicates that this substance is necessary for the rat. Euler and Malmberg ('36) fed rats on a basal vitamin B-free diet, supplemented by an acid clay adsorbate for vitamin B_1, riboflavin and an acid clay filtrate prepared from yeast, for other B vitamins. Over half of the experimental animals on this diet died, the remainder showed 'irregular' gains in weight, averaging 0.9 gm. per day. When 1.0 mg. of nicotinic acid amide was fed per day, only one animal out of nine died, the rest showed 'regular' gains in weight. Funk and Funk ('37) state that pigeons and rats fed on purified food ingredients plus thiamin, cod liver oil and vitamin B complex (U. S. Vit. Corp.) gave larger food intakes and better weight gains when nicotinic acid or the amide were fed than did the controls which received no nicotinic acid. Frost and Elvehjem ('37) obtained a definite growth response when nicotinic acid and adenine nucleotides were fed to rats on a vitamin B-deficient diet supplemented by thiamin and riboflavin. A dose of 1.0 mg. per day was necessary in order to produce a maximum effect.

More recently Gyorgy ('38) showed that the inclusion of 0.1% nicotinic acid in the diet of vitamin B_6 deficient rats prevented the appearance of panmyelophthisis described by Gyorgy et al. ('37). In view of this conflicting evidence it was thought desirable to reinvestigate the question as to whether nicotinic acid is essential for the rat or not.

The difficulty in such experiments is to supply the other factor in the vitamin B_2 complex free from nicotinic acid.

Previously maize has been given as a source of vitamin B_2 complex. The feeding of this foodstuff, however, raises the possibility that the animals may develop 'refection' due to the starch present in the maize and so synthesize nicotinic acid, in their intestinal tracts by bacterial action.

In these experiments, therefore, the use of starch has been avoided by feeding the following diet:

	%
Alcohol extracted casein	20
Lard	20
Sucrose	55
Salts	4
C.I.O.	1

The inclusion of this amount of lard in the ration delays the onset of vitamin B_6 deficiency (Birch, '38), so that one is able to keep animals alive on this diet for a long period when it is supplemented only by thiamin and riboflavin. Lard apparently has no sparing action on nicotinic acid deficiency as it has been repeatedly used in pellagra producing diets, Goldberger et al. ('28), Ruffin and Smith ('34).

This diet should be absolutely free from nicotinic acid and although deficient in other members of the vitamin B_2 complex, it was hoped that some symptoms of nicotinic acid deficiency, such as the panmyelophthisis described by Gyorgy et al. ('37) would show up before the animals died from other deficiencies. Nicotinic acid was fed to some of the animals at levels of 1.0 and 0.1 mg. per day. No significant difference was, however, seen between those receiving nicotinic acid and those not. Typical growth curves of some of the animals are shown in figure 2. The symptoms exhibited at death were: a sparse greasy fur which was covered with dried blood due to hemorrhage from the edges of the ears and the nose; acrodynia was seen in over 50% of the animals; respiratory infections were also a frequent cause of death.

These experiments show that complete deficiency of nicotinic acid in the rat for over 150 days is not accompanied by any specific physiological dysfunction such as is seen in dogs, swine or in man. They do not, however, exclude the possibility that nicotinic acid may be necessary for normal growth. This latter possibility is rendered unlikely by the finding that good growth may be obtained with rats on a vitamin B_2 complex deficient diet by feeding molasses (fig. 3). This material is apparently free from nicotinic acid as it has been used extensively in pellagra producing diets by Goldberger and Tanner ('25) and by Ruffin and Smith ('34).

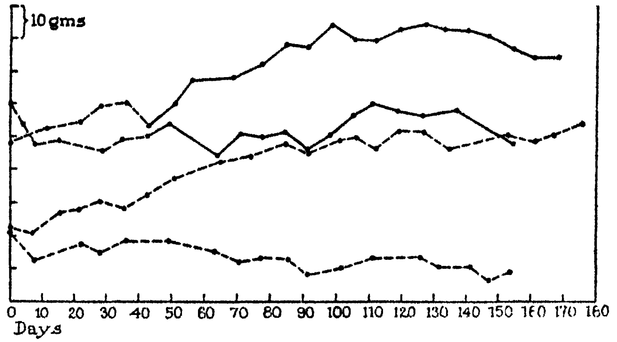

Fig. 2 Growth curves of rats on basal vitamin B-free diet containing 20% lard, supplemented by thiamin and riboflavin. Continuous lines indicate that nicotinic acid was also fed.

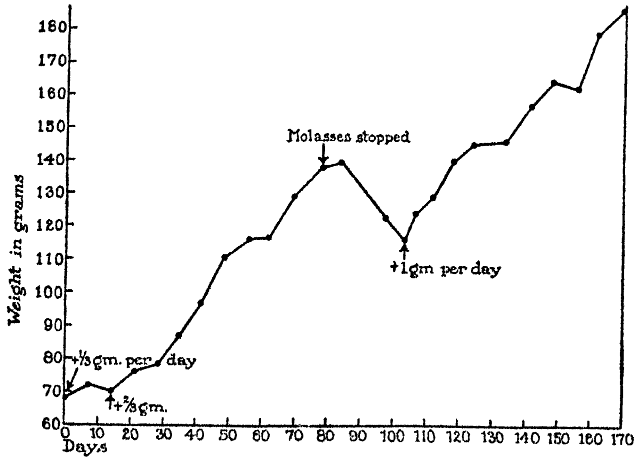

Fig. 3 Growth curve of rat on a diet consisting of casein 20%, sucrose 66%, butterfat 9%, salt mixture 4%, cod liver oil 1%, supplemented by thiamin and riboflavin. Molasses was fed in amounts indicated when the animal had developed acrodynia.

DISCUSSION

The most significant evidence that nicotinic acid or the amide are important factors in the nutrition of the rat is afforded by the work of Frost and Elvehjem who obtained a growth response of 20 gm. per week for 5 weeks when 1.0 mg. of nicotinic acid amide was fed per day. These workers showed that their factor W could be replaced by a mixture of nicotinic acid amide and adenine nucleotides. The subject is complicated, however, by the previous finding of Elvehjem, Koen and Oleson ('36) that factor W was different from the anti-blacktongue factor (now known to be nicotinic acid) and that it was thermolabile. Furthermore, when nicotinic acid amide was fed at the level of 80 micrograms per day (equivalent to 0.8 mg. per kilogram body weight) only a slight stimulation of growth ensued. Considering that 0.13 mg. per kilogram is sufficient to show a definite response in the dog which is extremely sensitive to nicotinic acid deficiency, 0.80 mg. per kilogram should be more than enough to produce a maximum effect in the rat if this substance were essential. It is, therefore, suggested that an interpretation different from that which involves the assumption that nicotinic acid is an essential substance for the rat may be used to explain the results of Frost and Elvehjem. For instance it is possible that the animals developed refection as 12% uncooked maize is fed in their diets.

With regard to the panmyelophthisis which Gyorgy ('38) is able to prevent by feeding nicotinic acid, it must be remembered, as Gyorgy himself pointed out, that no cures of this disease have been demonstrated. The present author has failed to produce this disease, and it is believed that other conditions which are not yet fully understood influence its appearance.

Details of Funk and Funk's work have not yet appeared and so it is therefore difficult to evaluate their findings. It is worthy of mention in connection with their work, that recently Carter and O'Brien ('38) have found that maize forms a complete diet for pigeons and addition of nicotinic acid had no weight restorative effect.

It appears, therefore, that the evidence in favor of the assumption that nicotinic acid is necessary for the rat is very inconclusive. In view of the failure of these present experiments to find any support for such a conclusion, it is reasonable to suppose that nicotinic acid is not necessary until more definite evidence is obtained for either view.

Attempts to estimate nicotinic acid by the dinitro chlorobenzene reagent used by Vilter et al. ('38) gave promising results when pure nicotinic acid was used, but the method was inapplicable to tissue extracts owing to impurities also giving colors which obscured that given by nicotinic acid. It was, therefore, impossible to determine if the tissues of rats on the nicotinic acid free diet contained this substance in normal amounts.

SUMMARY

PART I

The minimum dose of nicotinic acid necessary to protect the dog against blacktongue was found to be about 1.0 mg. per day for an animal of 5 to 10 kg. On a body weight basis 0.25 mg. of nicotinic acid per kilogram gave a rapid increase in weight and complete protection, 0.13 mg. per kilogram gave complete protection and a slight increase in weight, while 0.084 and 0.027 mg. per kilogram gave no protection. It has been calculated that 1.0 mg. of nicotinic acid is contained in 4 to 10 gm. of yeast.

PART II

Evidence is presented which indicates that nicotinic acid is not an essential substance for the rat.

I have pleasure in expressing my thanks to Dr. J. T. Wearn for the facilities afforded for this work, and to the S. M. A. Corporation and Messrs. Merck & Co. for gifts of riboflavin. The work was financed by the Bourne Fund in the department of medicine of Lakeside Hospital.

LITERATURE CITED

Birch, T. W. 1938 The relation between vitamin B_6 and the unsaturated fatty acid factor. J. Biol. Chem., vol. 124, p. 775.

Birch, T. W., H. Chick and J. C. Martin 1937 Experiments with pigs on a pellagra producing diet. Biochem. J., vol. 31, p. 2065.

Birch, T. W., P. Gyorgy and L. J. Harris 1935 The vitamin B_2 complex. Differentiation of the anti-blacktongue and 'P.– P.' factors from lactoflavine and vitamin B_6 (so-called 'rat pellagra' factor). Biochem. J., vol. 29, p. 2830.

Carter, C. W., and J. R. O'Brien 1938 Fractionation of Peter's concentrate in relation to pigeon nutrition. Chem. Ind., vol. 57, p. 470.

Chick, H., T. F. Macrae, A. J. P. Martin and C. J. Martin 1938 Curative action of nicotinic acid on pigs suffering from the effects of a diet consisting largely of maize. Biochem. J., vol. 32, p. 10.

Elvehjem, C. A., C. J. Koehn, Jr., and J. J. Oleson 1936 A new essential dietary factor. J. Biol. Chem., vol. 115, p. 707.

Elvehjem, C. A., R. J. Madden, F. M. Strong and D. W. Woolley 1937 Relation of nicotinic acid and nicotinic acid amide to canine blacktongue. J. Am. Chem. Soc., vol. 59, p. 1767.

———— 1938 The isolation and identification of the anti-blacktongue factor. J. Biol. Chem., vol. 123, p. 137.

Euler, H., and M. Malmberg 1936 Aktivatoren des Kohlenhydratabbaues als wasserlosiche Nahrungskomponenten. Biochem. Z., Bd. 284, S. 455.

Fouts, P. J., S. Lepkovsky, O. M. Helmer and T. H. Jukes 1937 Treatment of human pellagra with nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 405.

Frost, D. V., and C. A. Elvehjem 1937 Further studies on factor W. J. Biol. Chem., vol. 121, p. 255.

Funk, C., and I. C. Funk 1937 The value of pyridine derivatives in nutrition. J. Biol. Chem., vol. 119, p. xxxv.

Goldberger, J., and W. F. Tanner 1925 A study of the pellagra-preventive action of dried beans, casein, dried milk and brewer's yeast, with a consideration of the essential preventive factors involved. U. S. Pub. Health Rep., vol. 40, p. 54.

Goldberger, J., G. A. Wheeler, R. D. Lillie and L. M. Rogers 1928 Experimental black tongue and the blacktongue preventive in yeast. U. S. Pub. Health Rep., vol. 43, p. 657.

Gyorgy, P. 1938 Nicotinic acid and prevention of nutritional panmyelophthisis in rats. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 732.

Gyorgy, P., H. Goldblatt, F. R. Miller and R. P. Fulton 1937 Panmyelophthisis with hemorrhagic manifestations in rats on a nutritional basis. J. Exp. Med., vol. 66, p. 579.

Rhoades, C. P., and D. K. Miller 1935 Vitamin B_2(G) and canine blacktongue. Science, vol. 81, p. 159.

Ruffin, G. M., and D. T. Smith 1934 The treatment of pellagra with certain preparations of liver. Am. J. Med. Sci., vol. 187, p. 512.

SCHMIDT, H. L., JR., AND V. P. SYDENSTRICKER 1938 Nicotinic acid in the prevention of pellagra. J. Am. Med. Assoc., vol. 110, p. 2065.

SEBRELL, W. H., R. H. ONSTOTT, H. F. FRAZER AND F. S. DAFT 1938 Nicotinic acid in the prevention of blacktongue of dogs. J. Nutrition, vol. 16, p. 355.

SMITH, S. G., G. MARGOLIS AND L. H. MARGOLIS 1938 Effect of autoclaving on vitamin potency of nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 251.

SPIES, T. D., CLARK COOPER AND M. A. BLANKENHORN 1937 Therapeutic administration of nicotinic acid in human beings during health and disease. Read before the Central Soc. for Clin. Res. in Chicago.

STREET, H. R., AND G. R. COWGILL 1937 The cure of canine blacktongue with nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 547.

VITLER, S. P., T. D. SPIES AND A. P. MATHEWS 1938 The absence of nicotinic acid in the urine of pellagrins and a method for its quantitative estimation. J. Am. Chem. Soc., vol. 60, p. 731.

THE ENERGY CONTENT OF GOAT MILK[1]

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THREE FIGURES

(Received for publication November 7, 1938)

The purpose of this work was to study the correlation between energy value and some constituents of milk with the object of establishing ways of calculating the energy value instead of determining it directly.

The energy content of milk is important as an index of the food value of this constituent of the diet. The nutritive value of milk lies chiefly in its content of protein and energy not to mention the mineral matter and vitamins. From the standpoint of the efficiency of the milk goat as a transformer of food energy into milk energy, it is necessary to know what per cent of the food energy utilized appears as milk energy.

The experiments reported by Graham et al. ('38) show that the efficiency of the mammary gland is more than 90%. The calculations included the maintenance energy of the mammary tissue as well as the cost of the production of the milk and would therefore show that the cost of transference of blood precursors to milk constituents would be less than 10%.

The calorific value of cow's milk has been determined by a number of investigators. Overman and Sanmann ('26) determined the energy content of 212 samples of cows' milk. They found the correlation between fat percentage and calories per kilogram of milk to be $r = 0.9814 \pm 0.0017$, indicating that the energy content of the milk could be estimated with a high degree of accuracy from its weight and fat percentage.

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[1] Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series no. 582.

Overman, Sanmann and Wright ('29) reported the analysis of 1999 samples of milk. These data were studies by Overman and Gaines ('33) in conjunction with the 212 analyses of milk for energy residing in the various components of milk as follows in calories per gram: fat 9.312, protein 5.358, lactose 3.987, ash 4.980 and water —0.036. They did not explain the energy calculated to reside in the ash. They are of the opinion that all of the constituents should be considered and computed from the following formula.

$$E = 93.12f + 53.58p + 39.87l + 49.80a - 0.356w$$

where E is calories per kilogram of milk and f, p, l, a and w are the percentages of fat, protein, lactose, ash and water, respectively. These values vary to some extent depending on the number of components studied in calculating the values. The standard values reported by Abderhalden ('08) are fat 9.23, protein 5.71 and lactose 3.95 calories per gram. The value obtained by Overman and Gaines for fat agrees closely with the standard value, which is probably due to the greater proportion of the total energy being represented by fat and also to the accuracy of the fat determination.

During the course of experiments on the nutrition of the mammary gland of the goat, eighteen determinations of the energy value of the milk were made and compared with the values obtained by calculating the energy content from the composition of the milk. These values were compared with those of cow's milk of corresponding fat percentage.

The eighteen milk samples studied were obtained from four goats. Samples were taken at all stages during the normal lactation period from two of the goats.

Determinations of fat, total nitrogen, lactose, inorganic and total phosphorus, total solids and energy value were made for nearly all of the samples studied. The determinations were made as soon as the sample of milk was obtained and preservatives were not used. All of the determinations were made in duplicate with the exception of the energy determinations which were run in triplicate.

The percentage of fat was determined by the Babcock method. Total nitrogen was determined by the official Kjeldahl method (1934) and milk protein was obtained by multiplying the total nitrogen by 6.38. Total solids were determined in the usual way and the dried sample used for the determination of energy value (table 1).

TABLE 1

The composition of goat milk—by determination

GOAT NO.	NITROGEN	LACTOSE	FATS	SOLIDS	ENERGY PER 100 ML. OF MILK	ENERGY PER GRAM OF DRIED MILK	TOTAL PHOSPHORUS MILLIGRAM PER 100 ML.	INORGANIC PHOSPHORUS MILLIGRAM PER 100 ML.
	%	%	%	%				
348	0.59	6.02	4.80	19.12	114.00	5.96	......	62.76
348	0.65	5.24	5.25	16.11	94.04	5.84	......	63.98
348	....	5.25	5.00	18.68	111.53	5.97	118.35	57.70
30	0.67	4.63	6.45	17.28	100.95	5.84	132.70	75.09
30	0.66	5.56	4.20	15.95	86.79	5.44	161.00	94.47
30	0.76	4.89	....	14.68	83.02	5.66	126.40	92.37
30	0.51	....	5.40	14.99	85.74	5.72	142.80	92.27
30	0.45	4.78	6.40	16.35	97.26	5.95	140.40	....
30	0.70	5.09	3.80	12.79	70.30	5.50	117.50	....
403	0.53	4.87	4.35	14.37	82.25	5.72	106.40	64.87
403	0.47	5.00	4.50	14.04	80.97	5.77	95.70	52.53
403	0.49	5.37	3.90	14.10	76.47	5.42	91.50	65.40
403	0.46	4.69	6.50	16.10	91.71	5.70	102.50	68.30
403	0.52	4.93	4.50	14.44	78.74	5.45	104.70	59.90
403	0.64	5.29	5.00	14.58	71.53	4.90	......	....
403	0.39	4.98	2.30	10.61	53.46	5.04	144.78	....
403	0.47	....	6.00	15.52	91.83	5.92	......	....
536	0.49	4.31	3.40	12.51	68.37	5.47	......	....
Av.	0.56	5.06	4.80	15.12	85.50	5.63	121.9	....

The energy value was determined in an Emerson calorimeter equipped with a Daniels adiabatic jacket.[2]

There was no constant increase or decrease in the values for nitrogen, lactose, fat, total phosphorus, inorganic phosphorus or solids-not-fat with the advance of the stage of lactation.

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[2] The authors wish to thank Dr. S. Brody, Department of Dairy Husbandry, University of Missouri, for the use of the Emerson calorimeter.

The energy value, total solids, energy accounted for by non-lactose sources, and the number of milligrams of fat per calorie decreased with advanced lactation. The ratio of lactose to fat was very irregular. The number of determinations made was too small to show a definite ratio of phosphorus to lactose (table 1a).

TABLE 1a

The composition of goat milk (calculated relationships)

GOAT NO.	PROTEIN	NITROGEN PER CALORIE	FAT PER CALORIE	LACTOSE PER CALORIE	RATIO LACTOSE TO FAT	PHOSPHORUS TO LACTOSE	ENERGY FROM NON-LACTOSE SOURCE	ENERGY FROM NON-FAT SOURCE	ENERG FROM SOLIDS NOT-FA
	%	mg.	mg.	mg.					
348	3.77	5.19	43.45	52.85	1: 0.82	......	90	69	14.3
348	4.15	6.91	57.62	55.71	1: 1.03	......	71	45	10.8
348	....	....	46.27	47.09	1: 0.98	1: 44.4	91	65	13.7
30	4.29	6.66	65.94	45.84	1: 1.43	1: 34.8	83	41	10.9
30	4.19	7.57	49.94	64.03	1: 0.78	1: 34.5	65	48	11.7
30	4.85	9.16	....	59.92	......	1: 38.7	64	..	....
30	3.27	5.98	64.99	....	......	......	..	36	9.6
30	2.84	4.58	67.91	49.10	1: 1.38	1: 34.0	79	38	9.9
30	4.46	9.94	55.79	72.52	1: 0.77	1: 43.4	50	35	9.0
403	3.36	6.41	54.58	59.15	1: 0.92	1: 45.7	63	42	10.0
403	2.99	5.79	57.35	61.75	1: 0.92	1: 52.2	61	39	9.5
403	3.13	6.43	52.63	53.66	1: 0.98	1: 58.6	55	40	10.2
403	2.92	4.98	73.15	51.11	1: 1.43	1: 45.7	73	31	9.6
403	3.31	6.58	58.98	62.56	1: 0.94	1: 47.0	59	37	9.9
403	4.08	8.95	72.14	78.89	1: 0.97	......	52	25	9.6
403	2.52	7.38	44.40	93.22	1: 0.47	1: 34.4	33	32	8.3
403	2.97	5.07	67.43	....	......	......	..	36	....
536	3.15	7.21	50.34	63.09	1: 0.80	......	51	37	9.1
Av.	3.54	6.75	57.82	60.28	1: 0.96	......	..	..	10.4

The determined energy was compared with the calculated energy, using the values of Malcolm and Hall ('07–'08), fat 9.318, protein 5.860 and lactose 3.950 calories per gram, and also by using their value of 5650 calories per gram for total solids (table 2).

In table 2 it may be seen that the energy value of milk can be estimated with considerable accuracy by the use of protein, lactose and fat percentages and also by the use of the percentage of total solids. These two methods give very nearly the same value and both are slightly above the determined energy value.

The equation $y = a + bx$ was fitted to the fat percentages and energy values by the method of least squares. The curve of the equation is plotted in figure 1.

TABLE 2

Energy content of goat milk

SAMPLE NO.	BY DETERMINATION CALORIES PER 100 ML.	BY CALCULATION FROM PROTEIN, FAT AND LACTOSE	BY CALCULATION FROM MILK SOLIDS AT 5650 CALS. PER GRAM
1	114.00	90.597	108.028
2	94.04	93.936	91.021
4	100.95	103.527	97.632
5	86.79	85.650	90.117
8	97.26	95.158	92.377
9	70.30	81.689	72.263
10	82.25	79.458	81.190
11	80.97	79.202	79.326
12	76.47	75.893	79.665
13	91.71	96.203	90.965
14	78.74	80.800	81.586
15	71.53	91.394	82.377
16	53.46	55.869	59.946
18	68.37	67.164	70.680
Average	83.34	84.04	84.08

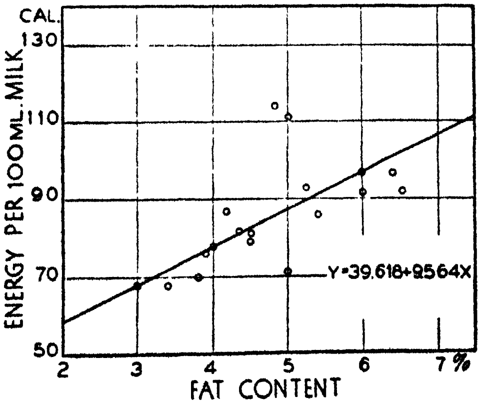

Fig. 1 Graph showing the calories per 100 ml. of milk of increasing fat percentage.

In table 3 comparison is made between the determined energy values and those calculated from the percentage of fat by the equation $y = 39.618 + 9.564x$. The results show that for fat percentages between 3.5 and 5.5 this equation gives the energy value with considerable accuracy.

The same equation was fitted to the total solids and energy values (fig. 2).

A comparison between the determined energy values and the calculated energy values obtained by the use of the equation indicates that the total energy content of goat milk can be estimated with a high degree of accuracy if the percentage of total solids is known.

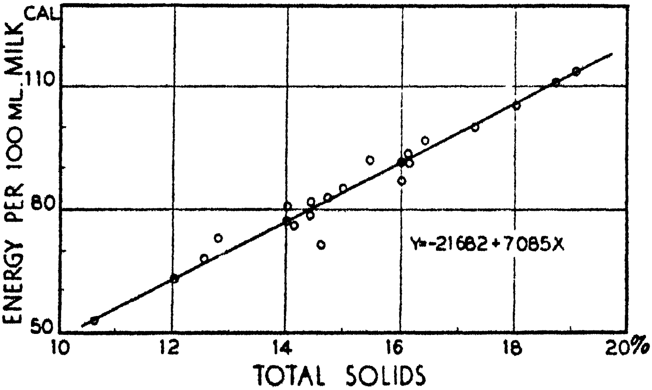

Fig. 2 Graph showing the relation between total solids and energy. This curve permits accurate estimation of energy when the total solids are known.

The results shown in tables 3 and 4 indicate that the percentage of total solids is a more reliable determination than the percentage of fat on which to calculate the energy value of goat milk.

Overman and Gaines ('33) found that the protein-energy ratio increased with an increase of fat percentage in the Holstein breed, while the Ayrshire breed showed a marked decrease in protein-energy ratio as the fat percentage increased.

TABLE 3

Relation of fat to energy content of goat milk

FAT	CALORIES PER 100 ML. DETERMINED	FAT	CALORIES PER 100 ML. CALCULATED [1]	DIFFERENCE
%		%		%
2.3	53.464	2.0	58.746	
3.4	68.368	2.5	63.528	
3.8	70.301	3.0	68.310	
3.9	76.468	3.5	73.092	+7
4.2	86.791	4.0	77.874	+2
4.35	82.255	4.5	82.656	+5
4.5	80.973	5.0	87.438	−21, +22
4.5	78.741	5.5	92.220	+7
4.8	113.998	6.0	97.002	+5
5.0	111.531	6.5	101.784	+11
5.0	71.531			
5.25	94.034			
5.4	85.744			
6.0	91.827			
6.4	97.262			
6.45	100.948			
6.5	91.708			

[1] $Y = 39.618 + 9.564x$.

TABLE 4

Relation of total solids to energy content of goat milk

TOTAL SOLIDS	ENERGY IN CALORIES DETERMINED PER 100 ML.	TOTAL SOLIDS	ENERGY VALUE CALCULATED PER 100 ML.[1]
%		%	
10.61	53.464	10.00	49.168
12.50	68.368	10.50	52.710
12.78	70.301	11.00	56.253
14.04	80.973	11.50	59.795
14.10	76.468	12.00	63.338
14.37	82.255	12.50	66.880
14.43	78.741	13.00	70.423
14.59	71.531	13.50	73.965
14.68	83.019	14.00	77.503
14.99	85.744	14.50	81.050
15.52	91.827	15.00	84.593
15.95	86.791	15.50	88.135
16.10	91.708	16.00	91.678
16.11	94.034	16.50	95.220
16.35	97.262	17.00	98.763
17.28	100.948	17.50	102.305
18.68	111.531	18.00	105.848
19.12	113.998	18.50	109.390
		19.00	112.933

[1] $y = 21.682 + 7.085x$.

In figure 3 the milligrams of nitrogen per calorie were plotted against fat percentage. The calculated equation shows that there is a definite decrease in the nitrogen-energy ratio with increase in fat percentage.

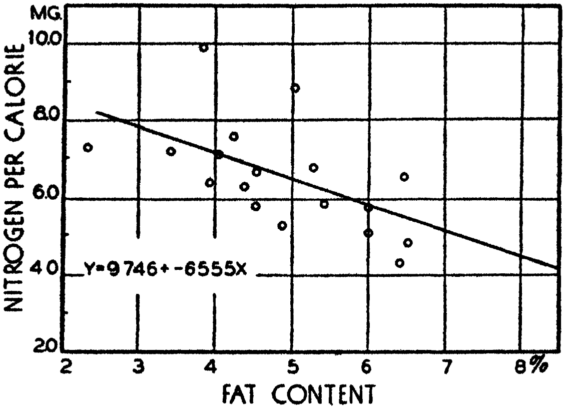

Fig. 3 Showing the definite decrease in protein-energy ratio with increase in fat percentage.

TABLE 5

Comparison of energy values of goat and cow milk

FAT (GOAT)	CALORIES PER POUND BASED ON EQUATION $y = 39.618 + 9.564x$ [1]	FAT (COW)	CALORIES PER POUND BASED ON EQUATION $y = 51.6M\ (2.66 + f)$ [2]
%		%	
3.0	300.24	3.0	292.06
3.5	321.26	3.5	317.85
4.0	342.28	4.0	343.66
4.5	363.30	4.5	369.46
5.0	384.32	5.0	395.26
5.5	405.33	5.5	421.06
6.0	426.35	6.0	446.85
6.5	447.37	6.5	472.66

[1] The equation $y = 39.618 + 9.564x$ gives the result in Calories per 100 ml. These results were converted into the corresponding value per pound (X4.3953).

[2] Gaines, 1928.

In table 5 comparison is made of the energy content of cow's milk with that of goat's milk containing the same fat percentage. Goat's milk containing from 3 to 5% compares favorably in energy content with cow's milk.

SUMMARY

The energy value, fat, nitrogen, lactose, inorganic and total phosphorus, and total solids were determined on eighteen samples of goat milk collected in various stages of lactation.

Comparisons were made between the determined energy value and that obtained by calculating from some constituents. It was found that the energy value could be estimated with considerable accuracy from either the protein, lactose and fat percentages or the percentage of total solids.

The results show that for fat percentages between 3.5 and 5.5 the equation $y = 39.618 + 9.564x$ gives energy values with considerable accuracy.

If the percentage of total solids is known the equation $y = 21.682 + 7.085x$ gives energy values nearly identical with the determined values.

One pound of 4% goat's milk was found to have an average energy value of 342.28 calories in comparison to 343.66 calories for cow's milk.

LITERATURE CITED

ABDERHALDEN, E. 1908 Textbook of physiological chemistry in thirty lectures. Translated by W. T. Hall and G. Defren, p. 722.

BRIGGS, A. P. 1922 A modification of the Bell Doisy phosphate method. J. Biol. Chem., vol. 53, p. 13.

GAINES, W. L. 1928 The energy basis of measuring milk yield in dairy cows. Bull. 308, Ill. Agr. Exp. Sta.

GRAHAM, W. R., JR., O. B. HOUCHIN, V. E. PETERSON AND C. W. TURNER 1938 The efficiency of the mammary gland in the production of milk. J. Physiol., vol. 122, p. 150.

MALCOLM, J., AND A. A. HALL 1907–1908 The heat value of milk as a test of its quality. J. Agr. Sci., vol. 2, p. 89.

OVERMAN, O. R., AND W. L. GAINES 1933 Milk energy formulas for various breeds of cattle. J. Agr. Res., vol. 46, p. 1109.

OVERMAN, O. R., AND F. P. SANMANN 1926 The energy value of milk as related to composition. Formulas for the computation of the energy. Bull. 282, Ill. Agr. Exp. Sta.

OVERMAN, O. R., F. P. SANMANN AND K. E. WRIGHT 1929 Studies of the composition of milk. Ill. Agr. Exp. Sta. Bull. 325.

SHAFFER, P. A., AND M. SOMOGYI 1933 Copper-iodometric reagents for sugar determination. J. Biol. Chem., vol. 100, p. 695.

STANDARD METHODS OF MILK ANALYSIS 1934 Sixth edition.

WHITEHORN, J. C. 1924–1925 A method for the determination of lipoid phosphorus in blood and plasma. J. Biol. Chem., vol. 62, p. 133.

A QUANTITATIVE BIOLOGICAL ASSAY OF VITAMIN K

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The interest in the fat-soluble antihemorrhagic factor or group of factors has grown rapidly during the past years and various methods have become available for the biological assay of the blood clotting agent, vitamin K. The Danish investigators Schønheyder, Dam et al. used a curative blood clotting method which was originally described by Schønheyder ('36, I, II) and has since been subjected to a thorough revision by Dam and Glavind ('38, I). This method of assay is based upon the observation that "animals suffering from vitamin K-deficiency become normal with reference to clotting time in 3 days when given sufficient food containing vitamin K." The California investigators Almquist and Stokstad ('37) described a preventive method which was later slightly modified by Klose et al. ('38); it is "based upon the occurrence of hemorrhage and upon simple measurement of blood clotting time."

It is interesting to note that the Danish method was criticized by the California workers (Almquist, '37) and that the California method appeared open to criticism to the Danish workers (Dam and Lewis, '37). Furthermore, the authors themselves have found fault with their own methods; Dam and Lewis ('37) observed a phenomenon resembling the refection as noted in vitamin B experiments; Dam and Glavind ('38, I) stated that, in spite of the precautions taken, they encountered animals with practically normal coagulation after months on the basal diet; and Almquist and Stokstad ('36) frequently noticed cases of spontaneous recovery. The method of the Danish workers was found to be difficult and time-consuming by Dann ('38), who adopted the preventive method of the California workers. Dann stated that this technic is "sufficiently accurate for comparative purposes and for a preliminary survey," in spite of the fact that she observed only a relatively small percentage of vitamin K-deficient chicks among her negative controls. Thayer and co-workers ('38) developed "a curative method of assay which appears to have certain advantages over methods previously used (Dam and Schønheyder; Almquist and Stokstad)"; chicks reared on a vitamin K-free diet were used; after marked deficiency had developed, the animals were given an active extract of the vitamin on 3 successive days and on the fourth day blood was drawn to determine the clotting time. Although seemingly valuable data have already appeared in the literature, Smith et al. ('38) believe that a critical survey of the chick assay technic should be made, "for obviously the dosage employed in both man and animals should be based upon accurate assay of the vitamin concentrates employed."

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[1] With the technical assistance of J. J. Savage and J. Spataro.

When the vitamin K investigations were started in this Institute, we first employed the curative technic of the Danish investigators. We soon found that a 3-day test period is unduly long, because the blood clotting power of the vitamin K-deficient chick can be rendered normal within a very few hours. This observation (Ansbacher, '38) served as a basis for the quantitative biological assay of vitamin K reported herein.

EXPERIMENTAL

The experimental animals employed were day-old single comb White Leghorn or White Rock chicks. They were housed in electrically heated brooders equipped with raised wire screen (2 mesh to the inch) bottoms; these brooders were kept in air conditioned rooms with a temperature of about 28°C. Tap water and the basal diets were fed ad libitum.

The basal rations used for the vitamin K assays are described in table 1. The basal protein in diet K-1 was fish meal which had been submitted to continuous extraction with ether for 7 days. The yeast used in the rations K-1 and K-4 had been extracted ten times with about 10 volumes of ether, then dried at about 50°C. and finally ground. The diet K-2 or K-3 was given to the day-old White Leghorn chicks for the first 4 days, then the diet K-1 was fed which is similar to the vitamin K-deficient diet E of Almquist and Stokstad ('36) and to the diet 417 of Dam and Glavind ('38, I). White Rock chicks received the diet K-1 as initial and sole basal diet because they were found to grow at a slightly greater rate than White Leghorn chicks. It should be noted that it is not necessary to start White Leghorns on diet K-2 or K-3; in our more recent experiments all the chicks were reared solely on diet K-1. The diet K-4 is identical with K-1 with the exception of the protein constituent which is Labco vitamin-free casein (Supplee et al., '36) in place of the fish meal of diet K-1; irrespective of the breed of the chicks, the ration K-4 was used as the initial and only basal diet.

TABLE 1

Composition of basal diets

ARTICLE OF DIET	RATION NO. K-1	RATION NO. K-2	RATION NO. K-3	RATION NO. K-4
	%	%	%	%
Cornstarch (dextrinized)	...	...	71	...
Polished rice (ground)	71	71	...	71
Crude casein (commercial)	...	17½	17½	...
Vitamin-free casein (Labco)[1]	...	...	...	17½
Fish meal[2] (ether extracted)	17½	...	...	...
Brewer's yeast (powdered)[3]	...	7½	7½	...
Brewer's yeast (ether extracted)	7½	...	...	7½
Salt mixture (Osborne and Mendel)[4]	3	3	3	3
Cod liver oil (Squibb)	1	1	1	1

[1] Distributed by the Borden Co., New York, N. Y.

[2] Lighthouse brand, distributed by Wilbur-Ellis Co., San Francisco, Calif.

[3] Strain L distributed by Anheuser-Busch, Inc., St. Louis, Mo.

[4] J. Biol. Chem., vol. 15, p. 317 ('13); 0.04% of copper sulfate and 5% of manganous sulfate were added to the salt mixture.

The incidence of the vitamin K-deficiency symptoms, resulting from the feeding of the various basal rations, is recorded in table 2. The hemorrhagic syndrome, typical for the vitamin K-avitaminosis of the chick, was most frequently found in the form of subcutaneous and intramuscular hemorrhages on the head, neck, breast, abdomen, back, wings and legs. The data presented show conclusively that diet K-1 contains little, if any, vitamin K. All the chicks on this diet died within 4 weeks unless they received a vitamin K supplement. It is also apparent that the diet K-3 contains a sufficient amount of vitamin K to prevent the occurrence of hemorrhages; therefore, it can be used only as an initial basal diet. The diet K-4, although identical with K-1 aside from the protein constituent, is evidently not suitable as a basal diet for vitamin K assays, in which a very pronounced deficiency is a prerequisite. Many chicks were found to be normal when this diet was used as the initial and sole basal ration for 23 days. Therefore, the Labco vitamin-free casein, the basal protein of this diet, either is not devoid of vitamin K or it interferes with the development of the hemorrhagic diathesis of the chick. Possibly prolonged extraction with ether of this brand of vitamin-free casein would result in a vitamin K-free product.

The data obtained from the feeding of the vitamin K-free ration K-1 are summarized in table 3. All the groups of experimental animals showing a numerical figure followed by K_2 received the ration K-2 as the initial diet and ration K-1 from the fifth day on; those marked 'K_3' received first the diet K-3, and after 4 days the ration K-1; those marked 'K_1' received the ration K-1 as the initial and sole diet. All the chicks were White Leghorns with the exception of those of lots 16, 17 and 18 which were White Rocks. The data of table 3 show that all the chicks became vitamin K-deficient within 15 days [2] and that only twenty-nine of the 1835 chicks

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[2] Since this paper was submitted for publication, twenty-one additional lots of chicks were used in vitamin K assays, namely 484 White Rocks and 2002 White Leghorns; all these animals became vitamin K-deficient within 15 days, bringing the number of normally responding chicks to 4321. However, 100 White Leghorns (lot 39) obtained from a different hatchery did not develop the hemorrhagic diathesis in the 15-day period. In collaboration with Dr. Sibylle Tolksdorf of this Institute we found that these chicks had enlarged thyroid glands; whether they had other anatomical abnormalities is not known. Attempts are being made to confirm these observations and experiments are under way to demonstrate a relationship of endocrine glands to vitamin K metabolism.

TABLE 2

Incidence of symptoms resulting from the feeding of the various basal rations

RATION	TOTAL NUMBER OF CHICKS	MORTALITY 1ST TO 10TH DAY	VISIBLE SYMPTOMS ON													23RD DAY NORMAL BLOOD CLOTTING TIME AND NO SYMPTOMS
			11th day	12th day	13th day	14th day	15th day	16th day	17th day	18th day	19th day	20th day	21st day	22nd day	23rd day	
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
K-1	267 [1]	5.6	40.1	20.9	16.5	7.5	9.4 [5]	...	...	...	...	..	..	..	..	...
K 2 or K-3 for 4 days, then K-1	1568 [2]	6.7	16.2	26.7	25.1	19.5	5.8 [6]	...	...	...	...	..	..	..	..	...
K-3 for 10 days, then K-1	100 [3]	4.0	...	...	6.0	9.0	16.0	14.0	20.0	18.0	13.0	..	..	..	..	...
K-4	115 [4]	2.6	2.6	10.5	17.4	1.7	8.7	19.1	1.7	3.5	3.5	5.2	0.9	2.6	4.3	15.7

[1] Composite of three lots.

[2] Composite of thirteen lots.

[3] Lot no. 11.

[4] Composite of five lots.

[5] Includes 1.9% with no visible symptoms, but with a coagulation time of more than 30 minutes.

[6] Includes 1.5% with no visible symptoms, but with a coagulation time of more than 30 minutes.

TABLE 3

Incidence and type of symptoms resulting from the feeding of the vitamin K-free diet (K-1)

LOT NO.	TOTAL NUMBER OF CHICKS	MORTALITY 1st to 10th day	VISIBLE SYMPTOMS ON					NO SYMPTOMS BUT BCT[2] > 30′	HEMORRHAGES ON								
			11th day	12th day	13th day	14th day	15th day		Abdomen	Legs	Shoulders	Wings	Head	Breast	Side	Back	Neck
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
5-K_2	200	6.5	27.0	30.0	17.0	19.5	...	..	25.0	15.0	6.5	14.0	10.5	8.0	..	7.0	7.5
6-K_2	100	8.0	...	25.0	36.0	21.0	10.0	..	28.0	14.0	12.0	14.0	6.0	6.0	3.0	8.0	1.0
7-K_3	103	3.9	22.4	30.1	25.2	18.4	...	..	35.8	17.5	6.8	9.7	6.8	7.8	1.0	9.7	1.0
8-K_3	103	9.7	28.2	35.9	17.5	8.7	...	..	33.0	17.5	5.8	8.8	8.8	7.8	2.9	3.8	1.9
9-K_2	101	3.0	...	24.7	44.6	27.7	...	..	30.7	13.9	12.8	15.7	3.0	13.9	4.0	2.0	1.0
10-K_2	204	8.8	26.0	29.4	22.1	13.7	...	..	21.6	12.7	17.2	10.8	8.3	9.3	6.4	3.9	1.0
13[1]-K_3	101	2.0	5.0	23.7	34.6	29.7	...	5.0	18.8	17.9	11.9	16.8	5.9	6.9	6.9	1.0	6.9
14-K_3	99	8.1	25.2	20.2	20.2	14.1	7.1	5.1	16.2	19.1	13.1	7.1	18.2	5.1	4.0	1.0	3.0
15-K_3	112	7.1	7.1	17.9	31.3	24.1	8.9	3.6	15.2	16.9	16.1	16.1	7.1	3.6	4.5	3.6	6.2
16-K_3	79	11.4	24.0	16.5	16.5	25.3	6.3	..	19.0	12.6	17.7	22.8	5.1	1.3	3.8	3.8	2.5
17-K_1	80	7.5	68.8	11.3	6.2	6.2	...	..	20.0	15.0	25.0	16.3	3.7	..	2.5	5.0	5.0
18-K_1	85	5.9	35.3	29.4	21.2	4.7	3.5	..	34.1	11.8	17.6	10.6	8.3	1.2	3.5	3.5	3.5
19-K_2	166	7.2	9.6	35.6	18.7	21.1	6.0	1.8	17.5	18.1	14.5	13.3	10.8	4.8	8.4	2.4	1.2
20-K_3	100	3.0	3.0	20.0	37.0	28.0	5.0	4.0	46.0	12.0	13.0	6.0	1.0	3.0	5.0	5.0	2.0
21-K_3	100	7.0	19.0	24.0	18.0	8.0	21.0	3.0	13.0	14.0	20.0	10.0	20.0	6.0	4.0	1.0	2.0
22-K_1	102	4.0	21.5	21.5	20.6	10.8	16.7	4.9	11.8	18.6	9.8	7.8	31.3	3.0	5.8	1.0	2.0
Chicks Total no.	1835	120	361	474	437	326	88	29	436	283	245	227	180	109	77	73	56
Per cent	100	6.5	19.7	25.8	23.8	17.8	4.8	1.6	23.8	15.4	13.3	12.4	9.8	5.9	4.2	4.0	3.1

[1] Lot no. 11, see table 2; the chicks of lot no. 12 were not used for vitamin K studies.

[2] BCT, blood clotting time.

did not show clinical signs of vitamin K-avitaminosis other than a prolonged blood clotting time (BCT > 30′).

The blood clotting time was determined in a manner similar to the one described by Almquist and Stokstad ('37): a small wing vein was punctured with a fine sewing needle; the blood was allowed to flow into a micro-test tube (3.8 × 0.2 mm.; 0.1 mm. thickness). The tube was placed at once in a water thermostat with a temperature of 38 to 40°C. and shaken by a constant speed mechanism. As the clotting time we considered the length of time necessary for a solid clot to form so that the tube can be inverted without dislodgement of the clot. The same definition for the clotting time has been given by Hawkins and Whipple ('35).

The coagulation time of the normal chick is less than 6 minutes and that of the chick showing the typical vitamin K-deficiency symptoms and receiving solely ration K-1 is markedly prolonged. Usually, the tubes were discarded after 30 minutes; however, in many experiments wherein the tubes were left in the thermostat for 3 hours, no clots were formed. In one set of experiments in which the tubes were left in the thermostat over night, no clots were observed within 15 hours.

The coagulation time of the chicks on ration K-1 is always markedly prolonged before hemorrhages appear. We did not observe a single animal on diet K-1 with a normal blood clotting time and with the hemorrhagic syndrome. The data of table 3 show also that there is only a relatively slight variation in the incidence of the hemorrhages from lot to lot. However, the site of the hemorrhages varies markedly from group to group and appears to be accidental.

As soon as a chick on ration K-1 shows the hemorrhagic syndrome, it is used for the assay of a vitamin K preparation. Its symptoms and weight are recorded. The test substance is then given orally by pipette. The pipette used is a ¼ ml. hypodermic syringe graduated in 1/100 ml. to which a glass tube (4 × 0.3 mm.; 0.1 mm. thickness) has been attached by means of rubber tubing. The content of the pipette is introduced into the chick's crop. The animal is then allowed to remain in a cage for 6 hours during which time it receives neither food nor water. Finally a blood sample is taken and the clotting time determined.

If the test substance contains vitamin K, the clotting power of the blood of the vitamin K-deficient chick will be restored within a very short time (Ansbacher, '38). All our tests are made in a 6-hour period, since we define the unit of vitamin K as the minimum amount necessary to render the blood clotting time of the vitamin K-deficient chick, weighing 70 to 100 gm., normal within 6 hours after administration. In the case of vitamin K concentrates, the product is fed in 0.10 ml. of cod liver oil.

Usually five chicks are employed for each concentration of the vitamin K preparation to be assayed, as shown in table 4 which records the biological activity of the vitamin K concentrate no. 14 prepared from alfalfa (Fernholz et al., '39). The data presented show that 1¼ mg. of this preparation is by definition equal to 1 unit of vitamin K. Parenthetically it may be added that the chicks fed the lowest dose of this concentrate did not die as a result of the vein puncture, although their clotting time was more than 30 minutes. They appeared to be much improved on the following day, but on the third day they were considerably weaker and death occurred within 5 days. The chicks to which the concentrate no. 14 had been given at the two highest levels showed a normal coagulation time for an entire week; after 12 days all these animals had a blood clotting time of more than 30 minutes and died from unchecked bleeding.

It should be noted also that the oil, used as a medium for administering vitamin K concentrates, appears to interfere to a small but noticeable degree with the potency of vitamin K. As shown in table 4, the biological activity of 1¼ mg. of the concentrate no. 14 fed in 0.05 ml. of cod liver oil was slightly greater than that of the same dose in 0.10 ml. This observation was made with many samples and the difference

TABLE 4

Biological activity of vitamin K contrate no. 14[1]

QUANTITY	62½ MG IN 0.25 ML. CLO[2]	50 MG. IN 0.20 ML. CLO	6¼ MG. IN 0.25 ML. CLO	5 MG. IN 0.20 ML. CLO	3¾ MG. IN 0 15 ML. CLO	2½ MG. IN 0.10 ML. CLO	2 MG IN 0 20 ML. CLO	1¾ MG. IN 0.10 ML. CLO	1½ MG. IN 0 15 ML. CLO	1½ MG. IN 0 10 ML. CLO	1¼ MG. IN 0.10 ML. CLO	1¼ MG. IN 0.05 ML. CLO	1 MG. IN 0.20 ML. CLO	1 MG. IN 0.15 ML. CLO	¾ MG. IN 0.15 ML. CLO	½ MG. IN 0.10 ML. CLO
Blood clotting time (minutes) of the experimental chicks after 6 hours	< ½	< ½	< ½	< ½	< ½	< ½	< ½	< 1	< 3	< 2	< 2	< 1	< 4	< 2	> 30	< 25
	< ½	< ½	< 1	< ½	< 1	< 1	< 3	< 3	< 4	< 5	< 6	< 1	< 8	< 5	> 30	> 30
	< ½	< ½	< 1	< ½	< 1	< 2	< 4	< 5	< 5	< 5	< 6	< 2	> 30	< 5	> 30	> 30
	< ½	< ½	< 2	< 1	< 2	< 2	< 4	< 6	< 5	< 5	< 8	< 3	> 30	< 15	> 30	> 30
	< 1	< 2	< 2	< 1	< 2	< 3	< 6	< 6	< 6	< 6	< 15	< 3	> 30	> 30	> 30	> 30

[1] The biological activity of the freshly prepared concentrate is shown. The vitamin K potency of concentrates gradually diminishes, and this particular sample was found to have lost about 50% of its activity during storage at a temperature of approximately 5°C. for 2 months.

[2] CLO, cod liver oil.

in potency observed was especially pronounced when 0.20 ml. of the oil was used as a vehicle in place of 0.10 ml.

In the earlier experiments we determined the blood clotting time of every chick showing the hemorrhagic syndrome. Usually, these determinations were made within 2 hours after the administration of a vitamin K preparation. Because we did not observe a single experimental animal with a normal coagulation time at this stage of the test, we omitted these determinations in the more recent assays. If a chick's coagulation time is to be determined twice during the experimental period, it is advisable to tap the left wing for the first blood clotting time determination and the right wing for the second one, since we observed a greater mortality from the puncture of a right wing vein. Vitamin K-deficient chicks usually die as a result of a vein puncture, unless they receive a potent vitamin K concentrate either shortly before or immediately after being bled.

To date more than 100 vitamin K samples have been tested by our biological assay method. The experience obtained in these determinations permits us to use a small number of experimental animals. The data of the individual chicks having received a preparation at a certain level, will give a clue as to where the concentration corresponding to about 1 unit of vitamin K lies. It was observed, namely, that the clotting time of most of the test animals will be reduced during the 6-hour test period to less than ½ minute, when several units of vitamin K are administered. Six hours after the feeding of 1 unit of vitamin K, the chicks are normal with respect to clotting, but one or two animals may be found with a slightly prolonged coagulation time of 6 to 15 minutes. If less than 1 but more than ½ unit of vitamin K is fed, several chicks will retain their prolonged clotting time. If ½ to ¼ unit is given, the clotting time remains prolonged during the 6-hour test period, but the animals will survive the vein puncture made in the determination of the coagulation power of the blood. If less than ¼ unit is administered, the clotting time will not be changed and the animals will die from unchecked bleeding.

In view of the numerous data published by the Danish investigators Schønheyder, Dam and co-workers, it seemed desirable to obtain comparable data of our unit, defined above, and that of Dam, who gave the following definition (Dam, '37): a unit of vitamin K is that amount which is to be administered to the test animal per gram of body weight in order to reduce its value $R \geqq 200$ to 1 ($R = \frac{K}{K_n}$, K equals concentration of the tissue extract (Dam and Glavind, '38, II) necessary to cause clotting of the blood plasma in 3 minutes; Kn equals the corresponding concentration for normal plasma). In order to enable us to make a comparison of the Dam unit of vitamin K with ours, Dr. H. Dam of the Biochemical Institute of the University of Copenhagen, Denmark, sent[3] to us samples of his vitamin K standard, "a selected dried spinach powder formed into tablets and stored under precautions securing stability." This standard preparation, found to contain 500 units of vitamin K per gram by Dam and Glavind ('38, I), was biologically assayed and the results are recorded in table 5. For the biological assay, the tablets were

TABLE 5

Biological data of vitamin K standard (500 Dam units per gram)

AMOUNT	BLOOD CLOTTING TIME OF INDIVIDUAL CHICKS 6 HOURS AFTER ORAL ADMINISTRATION					
mg.	*minutes*					
270[1]	< ½	< ½	< ½	< ½	< ½	< ½
100	< ½	< ½	< ½	< ½	< ½	< ½
40	< 2	< 2	< 4	< 6	< 7	< 8
38	< 1	< 1	< 2	< 6	< 10	< 25
35	< 1	< 2	< 8	< 20	> 30	> 30
34	< 3	< 4	> 30	> 30	> 30	> 30
30	< 5	< 6	> 30	> 30	> 30	> 30
25	< 8	> 30	> 30	> 30	> 30	> 30
10	> 30	> 30	> 30	> 30	> 30	> 30

[1] One tablet.

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[3] We wish to take this opportunity of thanking Doctor Dam for his kindness in sending us his vitamin K standard preparation.

cut with a knife into small pieces, and weighed amounts were introduced into the gullets of vitamin K-deficient chicks by means of small forceps and pushed into the crops. Finally the chicks were given 1 ml. of warm water and their blood clotting time was determined in the usual manner after 6 hours. It is apparent from the data of table 5 that 100 mg. of the standard contain several units, since the clotting time of the chicks was reduced to less than ½ minute within the 6-hour test period. The data as a whole show clearly that 40 mg. of the standard preparation have a potency of 1 unit of vitamin K. Therefore, the vitamin K standard contains 25 units per gram, and since the potency of 1 gm. was found to be 500 Dam units by the European workers, our unit is equal to 20 Dam units of vitamin K.

Clinical investigations are now being made with highly potent vitamin K concentrates prepared in this Institute. The dosage employed in these experiments is based upon the unit of vitamin K, as defined in this report.

SUMMARY

A comparative study of various basal diets for vitamin K assays was made on 2050 chicks.

A ration was found which permitted the development of severe vitamin K-deficiency in all the animals within 15 days.

A 6-hour curative blood clotting method for the determination of vitamin K was developed.

The unit of vitamin K was defined and the biological response resulting from the administration of unit amounts of vitamin K reported.

The unit, as defined, was found to equal 20 Dam units of vitamin K.

LITERATURE CITED

ALMQUIST, H. J. 1937 The anti-hemorrhagic vitamin. Poultry Sci., vol. 16, p. 166.

ALMQUIST, H. J., AND E. L. R. STOKSTAD 1936 Factors influencing the incidence of dietary hemorrhagic disease in chicks. J. Nutrition, vol. 12, p. 329.

———— 1937 Assay procedure for vitamin K (anti-hemorrhagic vitamin). J. Nutrition, vol. 14, p. 235.

ANSBACHER, S. 1938 New observations on the vitamin K deficiency of the chick. Science, vol. 88, p. 221.

DAM, H. 1937 Vitamin K, das fettlösliche antihämorrhagische Vitamin. Angew. Chemie, Bd. 50, S. 807.

DAM, H., AND J. GLAVIND 1938 I. Determination of vitamin K by the curative blood-clotting method. Biochem. J., vol. 32, p. 1018.

———— 1938 II. The clotting power of human and mammalian blood in relation to vitamin K. Acta Med. Scand., vol. 96, p. 108.

DAM, H., AND L. LEWIS 1937 The chemical concentration of vitamin K. Biochem. J., vol. 31, p. 17.

DANN, F. P. 1938 Vitamin K assays. Am. J. Physiol., vol. 123, p. 48.

FERNHOLZ, E., M. L. MOORE AND S. ANSBACHER 1939 Unpublished data from this Institute.

HAWKINS, W. B., AND G. H. WHIPPLE 1935 Bile fistulas and related abnormalities. Bleeding, osteoporosis, cholelithiasis and duodenal ulcers. J. Exp. Med., vol. 62, p. 599.

KLOSE, A. A., H. J. ALMQUIST AND E. MECCHI 1938 Properties of the antihemorrhagic vitamin (vitamin K). J. Biol. Chem., vol. 125, p. 681.

SCHØNHEYDER, F. 1936 I. The quantitative determination of vitamin K. Biochem. J., vol. 30, p. 890.

———— 1936 II. Nogle Undersøgelser over den biologiske Virkning og Standardiseringen af det antihaemorrhagiske Vitamin K. Thesis, Copenhagen, Nyt Nordisk Forlag; Arnold Busck.

SMITH, H. P., E. D. WARNER, K. M. BRINKHOUS AND W. H. SEEGERS 1938 Bleeding tendency and prothrombin deficiency in biliary fistula dogs: effect of feeding bile and vitamin K. J. Exp. Med., vol. 67, p. 911.

SUPPLEE, G. C., G. E. FLANIGAN, Z. M. HANFORD AND S. ANSBACHER 1936 Lactoflavin, a possible contaminant of vitamin-free diets. J. Biol. Chem., vol. 113, p. 787.

THAYER, S. A., D. W. MACCORQUODALE, R. W. MCKEE AND E. A. DOISY 1938 The biological assay of the antihemorrhagic factor (vitamin K). J. Biol. Chem., vol. 123, p. cxx.

AN EXPERIMENTAL DETERMINATION OF THE MINIMUM VITAMIN A REQUIREMENTS OF NORMAL ADULTS [1]

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TWO FIGURES

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Present knowledge of the vitamin A requirements of man has been summarized very recently in a review article by Booher ('38). Articles by Bessey and Wolbach ('38) and by Clausen ('38) summarizing the physiological functions of vitamin A and the pathological signs of vitamin A deficiency have also been published recently. The timeliness of these articles dismisses the need for extended review of the literature here.

The development of nutritional night-blindness is generally recognized as the earliest tangible sign of an inadequate intake of vitamin A. For a discussion of the chemical basis of vision, including the role of vitamin A in the visual processes, the reader is referred to articles by Hecht ('37), by Wald ('35), by Wald and Clark ('37) and by Krause and Sidwell ('38). The daily intake of vitamin A or carotene which will just prevent the appearance of night-blindness is a physiologic minimum requirement and does not provide for the accumulation of vitamin A reserves in the body. According to Guilbert, Miller and Hughes ('37), the physiologic minimum requirement of vitamin A for animals (cattle, sheep and swine) may be supplied by a daily intake of 25 to 30 micrograms (approximately 40 to 50 international units) of carotene, or by about 6 to 8 micrograms (about 18 to 24 units)[2] of vitamin A per kilogram of body weight. These levels of carotene or vitamin A intake sufficed for excellent gains in weight and for the maintenance of a thrifty physical appearance of the animals for indefinite periods, but did not provide any margin for storage of vitamin A in the liver nor for the needs of normal reproduction and lactation.

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[1] This research was supported by an appropriation from Bankhead-Jones funds (Bankhead-Jones Act of June 29, 1935).

Edmund and Clemmesen ('37) have reported a study on the vitamin A requirements of young adult men as carried out at the state prison in Nyborg (Denmark). They found that the fixed prison allowance which furnished an average of 1225 international units per day (about 17 to 19 units per kilogram of body weight) did not prevent seasonal oscillation in the power of visual distinction. Another group of young men who received the regular prison fare plus ½ liter of whole fluid milk daily for a 6-month (autumn and winter) experimental period showed normal powers of visual distinction at all seasons. It was estimated that this latter group received an average total of about 1400 international units of vitamin A (about 20 to 22 units per kilogram of body weight) per day. In view of the fact that the vitamin A intake was estimated for food consumed by groups of persons, it was not possible to determine precisely the individual vitamin A intake nor the variations in requirements.

EXPERIMENTAL

Five well-nourished adult subjects (two male and three female) employed at the Bureau of Home Economics served as experimental subjects for the determination of the minimum requirements of vitamin A and carotene.

The basal diet. Each subject received throughout the duration of the experiments (5 to 6 months) a basal weighed diet of ordinary food items adequate in all known food essentials except vitamin A, which was included in as small amounts as was practicable. Both the kinds and amounts of food were varied from day to day in order that any possible unusual effect of particular food combinations on the utilization of vitamin A or carotene might be minimized and in order that the subjects might be as contented with the diet as possible.

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[2] This conversion was made by the authors on the assumption that 1 gm. of pure vitamin A is equivalent to about 3,000,000 international units as indicated in the report of Holmes and Corbet (J. Am. Chem. Soc., vol. 59, p. 2042, 1937).

All the food was prepared and weighed in a special kitchen at the Bureau of Home Economics. The daily diets of the subjects were supplied in quantities which would satisfy their respective appetites and would provide adequate and practically constant calories, protein, fat, calcium, phosphorus, iron and the known vitamins. At the same time, the daily intake of vitamin A was kept at as low a level as possible. In order to maintain an adequate level of calcium and phosphorus, milk was of necessity included in the diet. Separated milk containing less than 0.02% butterfat which was used in this experiment is reported to contain vitamin A in traces only. However, it was found by biological assay with rats that appreciable amounts were present in such milk (about 80 units per quart). Certain other foods containing small quantities of vitamin A were included to give color and flavor to an otherwise pale and bland diet. Vitamin A-free bread[3] made of white flour, hydrogenated cottonseed oil, sugar and yeast was especially baked for use in these experiments.

The menus for the basal diet were chosen from the following list of foods:

Meats: Bacon, chicken, fish (lean), ham, lamb, pork and veal (all visible fat removed except in the case of bacon).

Vegetables: Asparagus (bleached), beans (dried), beets, cabbage (bleached), cauliflower, celery (bleached hearts), corn (white), cucumbers, mushrooms, onions, parsnips, potatoes (white) and turnips (white).

Fruits: Apples, cranberries, grapefruit, grape juice, grapes (white), lemons, pears and strawberries.

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[3] Prepared by Mrs. Adelaide Freeman, Bureau of Home Economics, U. S. Department of Agriculture.

Miscellaneous: Cocoa, coconut, hydrogenated cottonseed oil, egg white, flour (white), gelatin, honey, macaroni, milk (fat content less than 0.02%), margarine (made with vegetable oil and containing no added vitamin A), white rice, saltines, sugar and tapioca.

Dried baker's yeast was used to supplement the dietary supply of thiamin and riboflavin; small amounts of ferric ammonium citrate or ferrous carbonate were added to insure an abundance of iron; iodine was supplied in the iodized salt used for seasoning; either viosterol or irradiation with the mercury vapor lamp supplied vitamin D. Vitamin C was provided in liberal amounts in the fruits and vegetables.

Breakfast was the least variable of the meals consisting of grapefruit, toast, bacon, margarine, honey, skim milk and black coffee. A representative dinner would be: chicken, potatoes and margarine, cauliflower, a small portion of cranberry sauce, pears and skim milk. Supper: navy bean soup, saltines, a small serving of apple, celery, and nut salad with lemon juice dressing, cocoa and angel food cake.

The average composition of the daily food intakes of each subject is shown in table 1. Average figures for the composition of the food items were used in making the dietary calculations.

Measurements of dark adaptation. Before and during the beginning of the period of low vitamin A intake, from ten to fifteen tests of dark adaptation were made on each subject in order to establish curves of normal response. These tests were made with the visual adaptometer designed and described by Hecht and Shlaer ('38). Measurements made with this instrument have already appeared in an article by Haig, Hecht and Patek ('38) in connection with a study of the utilization of vitamin A concentrates by an individual suffering from cirrhosis of the liver.

The dark adaptation measurements were preceded by a 3-minute period of light adaptation. The brightness of the light-adapting screen located 50 cm. directly in front of the subject was 1400 millilamberts. The brightness of this screen and the length of the light adaptation period were constant for all tests.

The light used in the determination of threshold values emanated from a filament lamp, the brightness of which was calibrated at frequent intervals. Gross gradations of the light intensity were obtained by the use of suitable calibrated neutral filters. The finer gradations were made by means of a carefully calibrated, movable, photometric wedge. In order to make the difference between the rod curve and cone curve more pronounced, the light used for the test spot was restricted to the violet region of the spectrum by using a filter transmitting only wavelengths below 460 mμ. The duration of the light stimulus was limited to 0.2 second by means of a photographic shutter.

TABLE 1

Average composition of basal weighed diets adequate in all other respects except vitamin A (quantities refer to daily intakes)

SUBJECT	TOTAL CALORIES	PROTEIN	FAT	MINERALS[1]			VITAMINS[2]			
				Calcium	Phosphorus	Iron	A	Thiamin	Ascorbic acid	Riboflavin
		gm.	*gm.*	*gm*	*gm.*	*gm.*	*I.U.*	*I.U.*	*I U.*	*S.U.*
I	2380	66	74	1.13	1.37	0.026	93	600	2800	870
II	2420	89	90	1.13	1.44	0.026	103	590	3200	960
III	2260	72	76	1.22	1.39	0.025	91	610	2900	960
IV	2260	82	82	1.22	1.46	0.024	88	560	2800	900
V	2160	78	76	1.15	1.39	0.023	90	550	2300	890

[1] Ferric ammonium citrate and ferrous carbonate supplements mentioned in text are included in these averages.

[2] Values for vitamin A, thiamin and ascorbic acid are given in terms of international units, riboflavin values in terms of Sherman units. The thiamin and riboflavin supplements from the yeast are included in these averages. Vitamin D was supplied either by administration of viosterol or by irradiation of the skin with emanations from a mercury quartz lamp.

For subjects I, II and III, dark adaptation was measured at a position 7° from the fovea on the nasal side of the retina; for subject IV, the retinal image was central, and for subject V, the retinal image was placed at 15° temporal. In all cases, the test spot was a circle whose diameter subtended a 3° visual angle. On three subjects the tests were made regularly on the right eye, and on the others on the left eye. However, measurements made on both eyes during the period of night-blindness demonstrated that both retinas were equally affected by lack of vitamin A.

Three curves of the dark adaptation response for subject I showing the normal curve, the curve of response 36 days after the beginning of the period of low vitamin A intake a short time after definite signs of night-blindness appeared, and a curve determined 43 days after the beginning of the period of low vitamin A intake when a very marked degree of night-blindness had developed are shown in figure 1. It is apparent from this set of curves that both the cones and the rods are affected as the result of an inadequate intake of vitamin A. When the degree of night-blindness was most marked (curve C) the subject was subjectively aware of a restricted visual field. This observation was confirmed by actual measure-

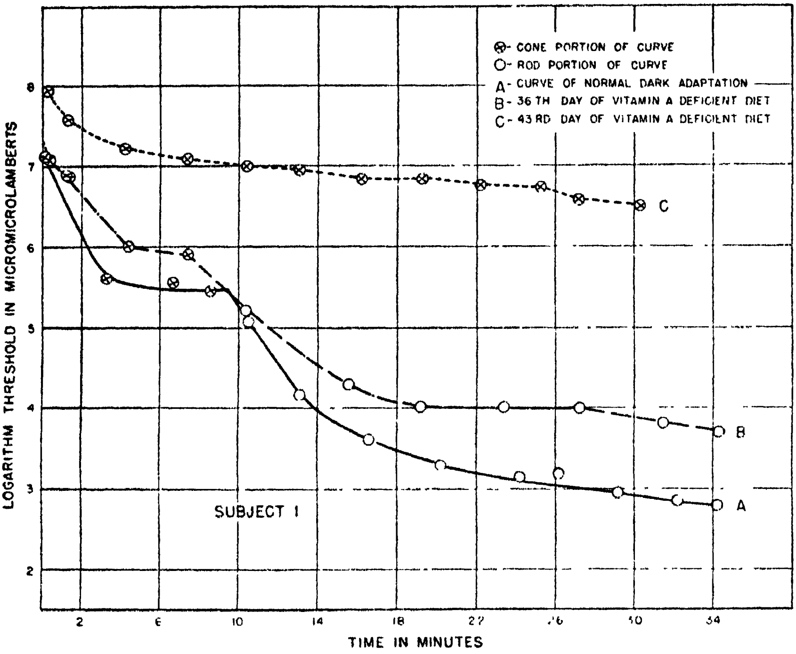

Fig. 1 Dark adaptation curves for subject I showing progress of night-blindness produced by the vitamin A-low diet.

ments[4] of the visual field with a perimeter. In comparison with this subject's normal visual field, the visual field area coexistent with the dark adaptation response represented by curve B (fig. 1) was perceptibly restricted. The magnitude of the restriction at this stage of vitamin A-deficiency, however, was not such as, of itself, would have definitely indicated an abnormal visual field area.

Ophthalmological examinations[5] made with a slit-lamp revealed no evidence of keratinization of the epithelial structures of the eye at any stage of night-blindness encountered in the course of these experiments.

TABLE 2

General description of experimental subjects

SUBJECT	SEX	AGE IN YEARS	HEIGHT IN CENTIMETERS	AVERAGE WEIGHT IN KILOGRAMS	AVERAGE NORMAL LIGHT THRESHOLDS[1] AFTER	
					6-minute dark adaptation	30-minute dark adaptation
					log micromicrolamberts	*log micromicrolamberts*
I	M	25	168.9	53.1±0.8	5.60±0.15	2.96±0.12
II	M	21	183.5	70.0±0.7	5.53±0.08	2.78±0.14
III	F	39	165.7	60.0±0.4	5.67±0.08	3.06±0.05
IV	F	30	163.8	51.6±0.6	5.87±0.15	3.87±0.20
V	F	40	164.5	59.7±0.4	5.85±0.13	2.83±0.10

[1] Subjects I, II and III were tested at a position 7° nasal; subject IV, central; subject V, 15° temporal.

Description of experimental subjects. A general description including sex, age, height, average body weight for the total experimental period and the normal light thresholds corresponding to 6- and to 30-minute periods of dark adaptation following a 3-minute period of light adaptation is given for each of the experimental subjects in table 2. The body weights shown in this table are averages of approximately weekly weighings, taken just before the noon meal. The male subjects were weighed without coats. The height and weight of all subjects were taken without shoes.

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[4] The visual field measurements were made by Dr. Louise Sloan, Wilmer Eye Clinic, Baltimore.

[5] These examinations were made by Drs. Alan Woods and William Rowland, Wilmer Eye Clinic, Baltimore.

Vitamin A value of cod liver oil and carotene supplements. The cod liver oil supplements used in these experiments were all taken from a single sample. The vitamin A value of this oil was 3000 international units per gram as determined by biological assay with rats using crystalline beta-carotene[6] (in benzene the $E^{1\%}_{1\,\mathrm{cm.}}$ values[7] at 430, 462 and 491 mμ were 1340, 2200 and 1930, respectively, as calculated from the equation $E^{1\%}_{1\,\mathrm{cm.}} = \frac{1}{c \times d} \log_{10} \frac{I_0}{I}$) as the standard of reference.

The vitamin A value of the commercial carotene used to supplement the diets was determined by careful biological assay[8] with rats using both pure beta-carotene and U.S.P. reference cod liver oil as standards of reference. According to this assay 0.6 microgram of the commercial carotene used induced growth in vitamin A assay rats not significantly different from that induced by 0.6 microgram of crystalline beta-carotene and/or 1 unit of U.S.P. reference cod liver oil. Chromatographic analysis[9] of the commercial carotene indicated that it contained 93.4% of carotene, of which 85% was beta-carotene and 15% was alpha-carotene. The remaining 6.6% of the material consisted of an unidentified pigment.

In order to effect complete solution of the commercial carotene in cottonseed oil so that precise aliquots could be weighed out for vitamin A assay rats and for the experimental subjects, the requisite amount of carotene was first dissolved in purified light petroleum ether (ligroin, boiling at 30° to 60°C.) and the cottonseed oil added subsequently. This solution (containing 60 to 80 mg. per 100 gm. of cottonseed oil) was then subjected to gradually diminishing pressures (temperature 25°C.) until the petroleum ether was all removed, as indicated by a constant weight of the solution. All carotene solutions were freshly prepared each week and stored at a temperature of 0°C. No loss of vitamin A activity in the concentrated carotene solutions stored under these experimental conditions could be detected by careful rat-growth assays.

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[6] The crystalline beta-carotene was purchased from the S. M. A. Corporation, Cleveland, Ohio.

[7] The spectrographic analyses of the beta-carotene were made by Mr. H. G. Wiseman, Bureau of Dairy Industry, U. S. Department of Agriculture.

[8] We are indebted to Rosemary Loughlin, Bureau of Home Economics, U. S. Department of Agriculture, for valuable assistance in making the biological assays of the carotene and cod liver oil supplements.

[9] The chromatographic analysis of the commercial carotene was made by Mr. L. A. Shinn, Bureau of Dairy Industry, U. S. Department of Agriculture.

Determination of vitamin A requirements. Subjects I to V showed unmistakable signs of impaired dark adaptation after 29, 27, 16, 124 and 39 days, respectively, on the vitamin A low diet which varied in vitamin A content only between the limits of about 90 and 100 international units per day. It is quite certain from these observations that ostensibly normal persons vary widely in bodily reserves of vitamin A. An increment of 0.5 log unit in the respective averages of the normal light threshold measurements, taken after 30 minutes dark adaptation, was taken as unmistakable evidence of induced hemeralopia. None of the subjects who showed this degree of hemeralopia ever showed threshold values within their respective normal limits unless vitamin A or carotene was administered.

When the subjects' average light threshold values following 30 minutes dark adaptation had attained values on the order of 1.0 log unit or more in excess of their respective normal threshold values, the administration of vitamin A active materials was begun. The time required for the development of this more marked degree of hemeralopia varied for the five subjects studied between the limits of 5 and 16 weeks on the vitamin A deficient diet. Each subject was continued on the vitamin A low diet supplemented either by vitamin A in the form of cod liver oil or by commercial carotene in cottonseed oil administered each day before lunch.

The first dose of cod liver oil, which was less than adequate to restore normal dark adaptation, nevertheless induced definite improvement in dark adaptation within 2 to 3 hours. This improvement was only temporary, however, and represented only a partial cure, and by the end of 24 hours the light threshold had risen appreciably, though not to the level preceding the cod liver oil administration. A given daily intake of cod liver oil less than was required for complete restoration of normal dark adaptation improved the light sense with each dose for a period of several days but finally reached a practically stationary state. Prolongation of administration of a given inadequate dose of vitamin A for a period of 2 to 3 weeks did not result in any further improvement in dark adaptation beyond that attained during the first several days. The daily vitamin A intake was not considered adequate until the subjects were able to maintain a light threshold within their respective normal limits at all times for a period of a week. It would seem, therefore, that the blood of vitamin A-deficient subjects maintained a level of vitamin A commensurate with the vitamin A supply in the food, or perhaps with the vitamin A content of the tissues maintained by that in the food.

The daily intake of vitamin A (in the form of cod liver oil plus the small vitamin A value supplied by the food) necessary for the maintenance of normal dark adaptation varied for the five subjects studied between the limits of 25 and 55 international units per kilogram of body weight.

Determination of carotene requirements. Subsequent to the determination of the minimum vitamin A requirements of four of the five experimental subjects in terms of international units supplied by cod liver oil, a similar procedure was followed in determining the minimum vitamin A requirements of these subjects in terms of crystalline carotene. For subject IV the vitamin A requirement was determined subsequent to the determination of the carotene requirement.

After establishment of definitely impaired dark adaptation by withholding or withdrawing all vitamin A intake except that carried by the basal vitamin A deficient diet, graded quantities of the crystalline carotene dissolved in cottonseed oil were administered to each subject. The minimum daily intake of carotene necessary for the support of normal dark

TABLE 3

Summary of data on the minimum vitamin A and carotene requirements per day for maintenance of normal dark adaptation

SUBJECT	IMPAIRED DARK ADAPTATION APPEARED AFTER	LIGHT THRESHOLD VALUES WHEN VITAMIN A SUPPLEMENTS WERE STARTED[1]		AVERAGE VITAMIN A VALUE OF BASAL DIET	DAILY REQUIREMENT FOR NORMAL DARK ADAPTATION						RATIO: CAROTENE REQUIREMENT
					Vitamin A			Carotene in cottonseed oil			
		6-minute dark adaptation	30-minute dark adaptation		Vitamin A value of cod liver oil suppl.	Total vitamin A intake	Vitamin A intake per kilogram	Vitamin A value of carotene suppl.	Total vitamin A intake	Vitamin A intake per kilogram	VITAMIN A REQUIREMENT
	days	*log micromicrolamberts*	*log micromicrolamberts*	*I.U.*	*I.U.*	*I.U.*	*I.U.*	*I.U.*	*I.U.*	*I.U.*	
I	29	7.05	no rod curves[2]	93	1800	1893	35.7	2950	3043	57.4	1.61
II	27	6.16	3.80	103	3800	3903	55.8	7080	7183	102.6	1.84
III	16	6.12	3.87	91	3050	3141	52.4	5310	5401	90.0	1.72
IV	124	6.10	4.74	88	1200	1288	24.8	2124	2212	42.5	1.71
V	39	6.00	3.55	90	1970	2060	34.3	3540	3630	60.5	1.76

[1] Subjects I, II and III were tested at a position 7° nasal; subject IV, central; subject V, 15° temporal.

[2] As shown in figure 1 (curve C) the rod structures were not functioning during the period of very marked impairment of dark adaptation.

adaptation was determined for each subject in accordance with the procedure described for the determination of physiologic minimum requirements for vitamin A in the form of cod liver oil.

The daily intake of carotene (expressed in terms of international units of vitamin A) necessary for the maintenance of normal dark adaptation varied for the five subjects studied between the limits of 43 and 103 units per kilogram of body weight.

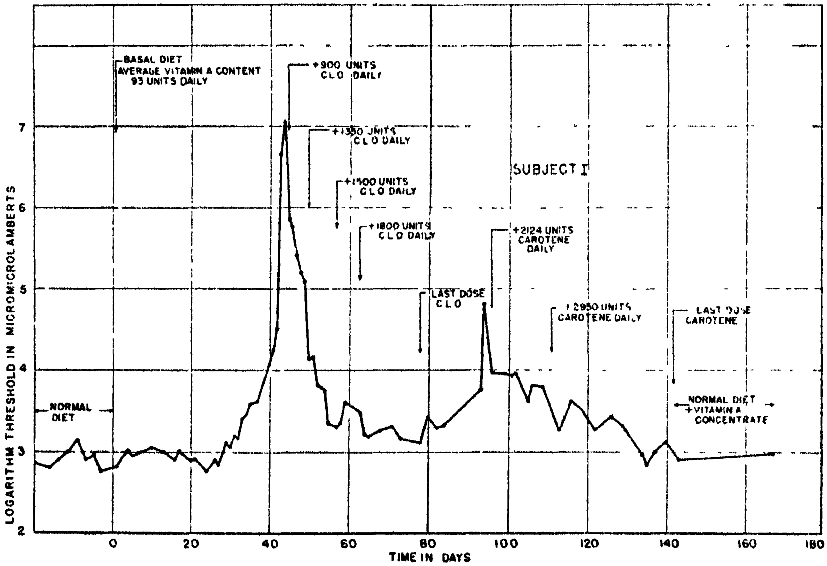

Fig. 2 Graphic representation of the experimental procedure followed in determining the minimum vitamin A and carotene requirements. Data shown refer to subject I. Ordinates refer to threshold values after 30 minutes of dark adaptation. Vitamin units refer in all cases to international unit values.

The experimental procedure involved in determining the minimum vitamin A and carotene requirements of the five subjects is depicted in chronological detail for one subject (subject I) in figure 2. The minimum vitamin A and carotene requirements, respectively, for each subject are summarized in table 3.

DISCUSSION

It is quite evident that the physiologic minimum requirements for vitamin A and/or carotene vary considerably among ostensibly normal adults. However, the ratios of the units of carotene to the units of vitamin A, respectively, which were required for normal dark adaptation in these subjects were almost constant (table 3). Unit for unit, the carotene in cottonseed oil was only about 50 to 60% as efficient in maintaining normal dark adaptation as vitamin A in the form of cod liver oil. In other words, the vitamin A potency of carotene in cottonseed oil, as determined by rat-growth assays, is only about 60% efficient in supporting dark adaptation in humans. Whether this represents a species difference in the absorption of carotene, or a species difference in the conversion of carotene into vitamin A, or a difference in the utilization of carotene for growth and dark adaptation was not indicated by the present experiments.

The human adult would appear to require somewhat higher absolute levels of either vitamin A or carotene per unit of body weight than cattle, sheep or swine. However, the estimates of the minimum vitamin A requirements of the domestic farm animals as determined by Guilbert et al. ('37) were based on needs for the prevention of gross signs of night-blindness, while those of human adults are based on needs for the prevention of a lesser degree of impaired dark adaptation. This explanation of the higher requirement levels for the human subjects reported here as compared with those reported for cattle, sheep and swine has ample experimental support. The levels of either vitamin A or carotene required to restore grossly impaired dark adaptation to a state which would appear normal with a less sensitive criterion (the biophotometer, for example) was on the order of 20% less than was required for restoration of a completely normal cone-rod dark adaptation response as measured with the visual adaptometer.

Measurements of dark adaptation were made with the biophotometer according to the method outlined by Jeans, Blanchard and Zentmire ('37) on the experimental subjects at various stages of vitamin A deficiency. Only marked degrees of night-blindness could be detected with this instrument, an explanation for which may be seen by reference to figure 1. Curves B and C in this figure clearly depict a moderate and a marked degree of impaired dark adaptation, respectively, as measured with the visual adaptometer. The 'critical measurements,' as made with the biophotometer, correspond to the readings shown in the three curves taken immediately (within a 20-second interval) after the light adaptation period. It is evident that the light threshold value measured within this time interval is not essentially different in curve B from that in the normal curve (curve A).

SUMMARY

Five adult subjects maintained on weighed diets adequate in all known food essentials except vitamin A (the average daily intake of which was not greater than 103 international units) showed unmistakable signs of impaired dark adaptation after 16, 27, 29, 39 and 124 days, respectively.

Impaired dark adaptation as measured with the visual adaptometer was the earliest definite ocular abnormality observed as a result of vitamin A deficiency.

The daily intake of vitamin A (in the form of cod liver oil plus the small vitamin A value supplied by the food) necessary for the maintenance of normal dark adaptation varied for the five subjects studied between the limits of 25 and 55 international units per kilogram of body weight.

The daily intake of carotene (expressed in terms of international units of vitamin A) necessary for the maintenance of normal dark adaptation varied for the five subjects studied between the limits of 43 and 103 units per kilogram of body weight.

The ratios of the requirements in terms of international units of carotene to the requirements in terms of international units of vitamin A in the form of cod liver oil were remarkably constant for all subjects. Unit for unit the carotene in cottonseed oil was about 50 to 60% as effective in supporting normal dark adaptation as vitamin A in the form of cod liver oil.

ACKNOWLEDGMENTS

We are especially indebted to Dr. Selig Hecht and his associates at Columbia University for the timely introduction of the visual adaptometer and for their continued interest in this research, and to our many associates at the Bureau of Home Economics who have served as experimental subjects or who have contributed to the culmination of this work in a variety of ways.

LITERATURE CITED

Bessey, O. A., and S. B. Wolbach 1938 Vitamin A physiology and pathology. J. Am. Med. Assoc., vol. 110, p. 2072.

Booher, L. E. 1938 Vitamin A requirements and practical recommendations for vitamin A intake. J. Am. Med. Assoc., vol. 110, p. 1920.

Clausen, S. W. 1938 The pharmacology and therapeutics of vitamin A. J. Am. Med. Assoc., vol. 111, p. 144.

Edmund, C., and S. Clemmesen 1937 On deficiency of A vitamin and visual adaptation. II (translated by Mrs. Clara Packness), London, Oxford University Press.

Guilbert, H. R., R. F. Miller and E. H. Hughes 1937 The minimum vitamin A and carotene requirement of cattle, sheep and swine. J. Nutrition, vol. 13, p. 543.

Haig, C., S. Hecht and A. J. Patek 1938 Vitamin A and rod-cone dark adaptation in cirrhosis of the liver. Science, vol. 87, p. 534.

Hecht, S. 1937 Rods, cones and the chemical basis of vision. Physiol. Rev., vol. 17, p. 239.

Hecht, S., and S. Shlaer 1938 J. Opt. Soc. Am., vol. 28, p. 269.

Jeans, P. C., E. Blanchard and Z. Zentmire 1937 Dark adaptation and vitamin A. J. Am. Med. Assoc., vol. 108, p. 451.

Krause, A. C., and A. E. Sidwell, Jr. 1938 The absorption spectra of visual purple and its photodecomposition products. Am. J. Physiol., vol. 121, p. 215.

Wald, G. 1935 Carotenoids and the visual cycle. J. Gen. Physiol., vol. 19, p. 351.

Wald, G., and A. Clark 1937 Visual adaptation and chemistry of the rods J. Gen. Physiol., vol. 21, p. 93.

FLUOROSIS IN RATS DUE TO CONTAMINATION WITH FLUORINE OF COMMERCIAL CASEIN. THE EFFECTS OF DARKNESS AND OF CONTROLLED RADIATION UPON THE PATHOLOGY OF THE TEETH[1]

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FIVE FIGURES

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INTRODUCTION

During the past 18 months, October, 1936 to April, 1938, studies have been made of the effects of isolated radiation in the visible and infra red spectrum upon growth and reproduction in three succeeding generations of rats. Three rooms were designed in which it was possible to give to rats continuous exposure to radiation of approximately equal energy in the visible, the near infra red and the far infra red regions of the spectrum. A fourth room was kept completely dark. All rooms were kept at constant temperature. A full description of these rooms will be given in a later paper. It is the purpose of this paper to describe certain abnormalities of the teeth which were observed in one group of rats.

Two standard diets were used in these experiments: the Sherman B diet (Sherman and Campbell, '24) and also the Standard Diet no. 1 of McCollum (Evans, '22).

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[1] Aided by a grant from the Committee on the Effects of Radiation upon Living Organisms, Divisions of Biology and Agriculture, National Research Council, and by a grant from the Carnegie Corporation of New York.

No attention was paid, until the experiments had been in progress for 1 year, to the teeth of the rats. They then obtruded themselves on our notice by the observation that certain rats were caught, by their upper incisors, upon the wires of the cage and were unable to extricate themselves. Examination showed that serious abnormalities of the teeth were developing in all the rats fed on the 'McCollum' diet (see footnote p. 336), while the teeth of a large group fed on the Sherman B diet were completely normal. It became evident that the abnormalities observed fitted the picture of fluorine poisoning described by McCollum ('25), Smith and Lantz ('33) and other writers though it was not at all obvious to us at first how the rats, which were fed on what we considered a standard normal diet, could have developed fluorosis.

EXAMINATION OF DIET INGREDIENTS AND OF THE TEETH OF THE RATS FOR FLUORINE

a. Diet ingredients. In comparing the two diets,

Sherman B		*McCollum no 1*	*per cent*
Whole wheat flour	2/3	Whole wheat flour	67.5
Klim	1/3	Casein	15.0
NaCl	2% of the whole wheat flour	Klim	10.0
		NaCl	1.0
		$CaCO_3$	1.5
		Butterfat	5.0

it is evident that only three ingredients are present in the 'McCollum' diet which are not used in the Sherman B mixture—namely casein, $CaCO_3$ and butterfat.

Spectrographic analyses of these three materials were carried out by Dr. L. T. Steadman of the division of radiology, department of medicine, School of Medicine and Dentistry. The technic of Churchill ('31) was adopted. By comparing the spectra of a series of standards with the casein spectrum (fig. 1), it was shown that the casein contained approximately 0.1% fluorine. This figure was adjudged to represent the true value within a factor of 2.

Because the contamination of fluorine in the casein was so marked, it seemed advisable to obtain chemical analyses as

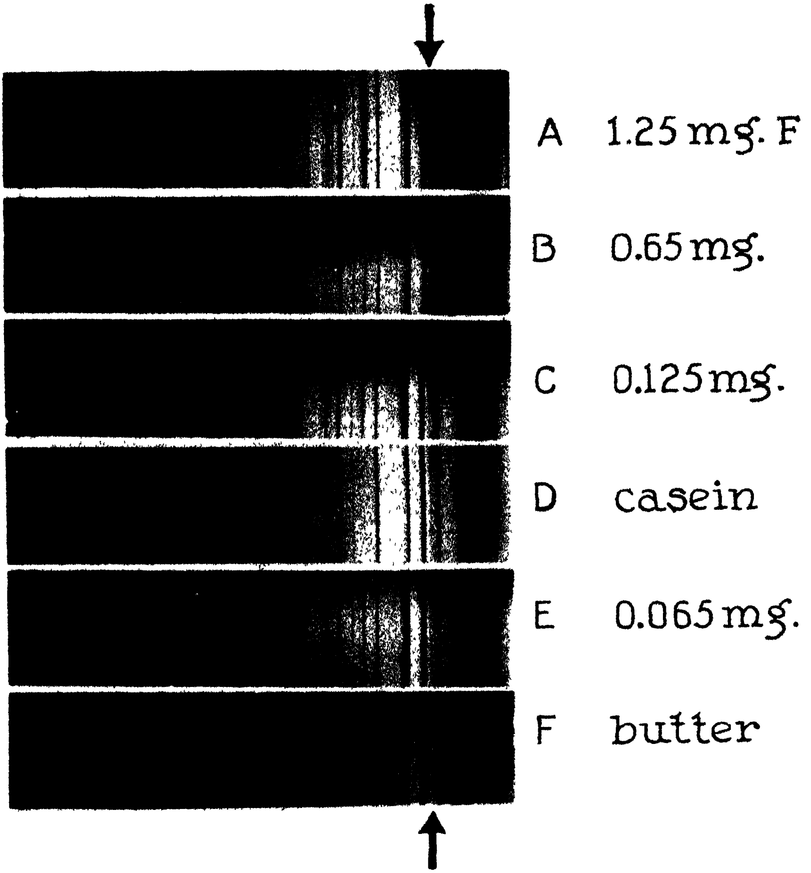

Fig. 1 Spectra showing CaF_2 bands at 5291 Å (between the arrow points). The spectra of a series of standards composed of fluorine-free whole powdered milk and varying amounts of NaF (A, B, C and E) may be compared with casein (D). The band is very dark in A, very light in E and absent in F (butter).

a check on the spectrographic data. Dr. G. H. Ellis of the Cornell University Agriculture Experiment Station kindly furnished these analyses using the methods of Willard and Winter ('33) and of Sanchis ('34). The results are as follows:

G. casein sample	*G. CaO added*	*Per cent fluorine*
10	1	0.20
5	1	0.19
5	0.5	0.19

Since the diet contained 15% of casein, the approximate fluorine content of the diet must have been 0.03 %. We have no means of knowing whether each batch of casein used during the year had been similarly contaminated, but if it had been, a rat ingesting 10 gm. of this diet[2] per day would obtain a daily dosage of 3 mg. of fluorine.

b. Teeth of rats. The incisors were taken from the jaws of ten rats, five with marked deformities which had been fed the 'McCollum' diet and five with normal teeth which had received the Sherman diet. The upper incisors and lower incisors were pooled for each diet and the enamel separated from the dentine by the centrifugal flotation method (Manly and Hodge, in press). Fluorine anlyses were made on these samples in duplicate or triplicate by Dr. W. D. Armstrong of the University of Minnesota using his own method (Armstrong, '36). The results are given in table 1. The normal values are similar to those recently reported by various workers, viz., about 0.01% fluorine (Bowes and Murray, '35; Gassmann, '08; Muñoz, '36). If the estimate of Ellis and Maynard ('36) is accepted, then the fluorine content of the teeth of 'normal' rats results from the ingestion of a diet containing 3 parts per million of fluorine. The fluorine content for the dentine and enamel of the fluoride poisoned rats is suprisingly high, in fact, ten to twenty-five times the normal content and about twice the values reported by Muñoz for fluoride poisoned lower incisors.

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[2] It should be remembered that although the diet used is designated 'McCollum' such fluorine contamination of the casein as we have described makes the diet pathological, an effect never observed by its author. For this reason, we have throughout the paper put the title McCollum in quotation marks, to signify the diet plus its contamination. Dr. E. V. McCollum was kind enough to read our manuscript before publication. The casein used in his stock breeding diet is not obtained from the same source as that used by us, and he states "In no instance have we seen any evidence of fluorosis in our stock animals. There is no discolouration of the teeth and no abnormal tooth structure such as is characteristic of fluorosis."

TABLE 1

Fluorine content of normal and fluoride poisoned incisors (data by Dr. W. D. Armstrong)

Incisor	Tissue	NORMAL Per cent fluorine	FLUOROSIS Per cent fluorine
Upper			0.143
	Enamel	0.0079	0.135
	Dentine	0.0107	0.264[1]
		0.0108	0.251
			0.253
Lower	Enamel	0.0087	0.117
		0.0083	
	Dentine	0.0111	0.257
		0.0111	0.264
			0.252[2]

[1] Average of two titrations at different times.

[2] Double distillation.

It should be noted in this table that the fluorine content of the dentine is higher than that of the enamel in both normal and abnormal teeth and is higher in approximately the same proportion in both groups.

MEASUREMENTS OF THE LENGTH OF THE TEETH

The skulls of a total of 130 rats of different ages and from three succeding generations, taken in approximately equal numbers from the four rooms, were cleaned and mounted. Of these eleven were the skulls of normals rats fed the Sherman B diet; the remainder, the skulls of abnormal rats fed the 'McCollum' diet.

Throughout our measurements the eleven Sherman B rats have been used as the basis of comparison with the groups taken from the different rooms. In the accompanying figures 3 and 4 the results for room I (dark room) and room III (visible room) have been plotted separately. Rooms II and IV near and far infra red rooms) have been combined.

The lengths of both upper and lower incisors were measured in the following way. The skull, magnified thirteen times, was projected by means of an opaque projector on to a screen. The curvature of the tooth was then fitted to its appropriate circle on polar coordinate paper. From the observed degree subtended by the arc of the circle covered by the tooth the length was calculated by the following formula:

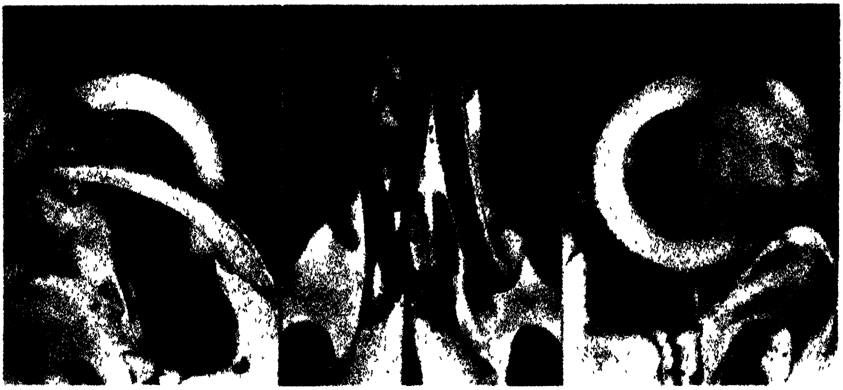

Fig. 2 A single skull shows the two principal tooth length abnormalities observed in fluorosis. On the left, an extreme overgrowth of the lower incisor has produced severe trauma of the mandibular bone. On the right is shown the more typical overgowth of the upper incisor. The center shows the right upper incisor in apposition with the left lower. The striae in the enamel are typical of fluorine.

$$L = \frac{\pi R D}{180} = 0.01745 \text{ R D mm.},$$

where R = radius and D = the number of degrees. The result was corrected for magnification.

We have expressed the results of these measurements in terms of the ratio between the length of lower incisors to the length of upper incisors on the right and the left side. Normally these ratios are fairly constant with age. Donaldson's figure expressed in this ratio is 1.3. Our results in normal rats follow his figures very closely. We have therefore taken this ratio of 1.3 as being normal.

Figure 3 shows the ratios obtained in the four rooms in rats fed on the 'McCollum' as compared with those on the Sherman B diet. A ratio markedly lower than normal except in the youngest radiated rats is at once apparent. This is due to a relative overgrowth of the upper incisors. In this series we have excluded four rats whose teeth showed such marked deformities as would render the ratios meaningless.

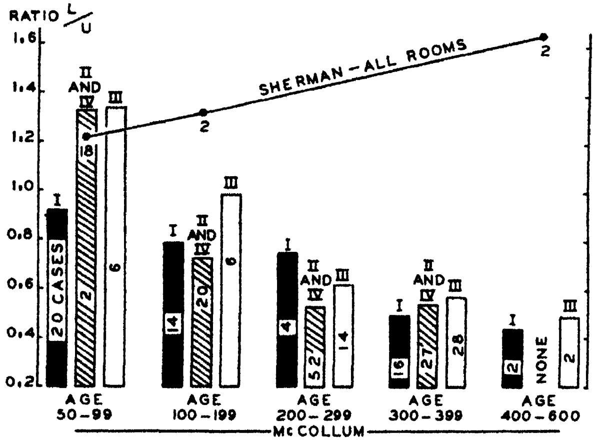

Fig. 3 Measurements of length of teeth. Sherman, normal diet; 'McCollum,' diet plus fluorine; III, animals exposed to visible radiation; II and IV, animals exposed to near and far infra red radiation; I, animals kept in darkness.

Such an example is shown in figure 2. In this rat the right upper incisor is in apposition to the left lower, with the results that the left upper and right lower incisors show unimpeded growth. With the exception of these four rats there was good enough apposition between right and left upper and lower incisors to justify expressing the results in terms of this ratio.

Differences in ratios possibly due to radiation. A comparison of the ratios for length of teeth figure 3 indicates the possibility that the presence of fluorosis in the rats receiving radiation has in some way made evident, in the early stages of the disease, some effect of light. We shall discuss this more fully in a later paper. It should, however, be noted that normal ratios were obtained in all our radiated rats in the earliest age group and that those receiving no radiation in the same age group showed the effect of fluorosis with lowered ratios. We have, it is true, fewer observations in the radiated than in the non-radiated group, so we cannot at this stage emphasize the significance of this observation.

MEASUREMENTS OF THE ERUPTION RATE OF TEETH IN RATS FED ON THE TWO DIETS

Weekly measurements of the rate of growth of the teeth of young rats fed on both diets were kindly undertaken by Dr. B. J. Frey after we had discovered the appearance of fluorosis in the colony. His results are of such great interest that they will be published by him in detail in a separate paper. These results, where the rate of growth of the teeth is plotted against time show a remarkably slower rate of growth in the teeth of the rats fed the 'McCollum,' as compared with those fed the Sherman B diet, which confirms the preliminary findings of Smith and Lantz ('35). Doctor Frey found that the divergence in the rate of growth on the two diets is more marked in the upper than in the lower incisors, though quite definite in both. Smith and Lantz have described a lower phosphatase content in the incisors of fluoride-fed rats. It would be of considerable theoretical interest to compare the phosphatase contents of the upper and the lower incisors of normal and fluoride fed rats, in view of the markedly decreased eruption rate for the upper incisors and the slightly decreased rate for the lowers in fluorosis.

ESTIMATION OF THE DEGREE OF COLOR OF THE TEETH

To measure the degree of color of the teeth we used a somewhat rough and arbitrary method. We employed an artist to paint, on glazed paper, degrees of color ranging from the normal, a red yellow, to the most bleached, almost white, teeth in our group and to connect these two extremes by gradations of color.

In this way eight units of varying color strength or chroma were obtained. The Munsell values measured on the Bausch and Lomb color analyzer were obtained through the kindness of Mr. J. W. Forrest of the Bausch and Lomb Company. The Munsell values for the eight units are as follows:

Unit	*Munsell Number*	*Unit*	*Munsell Number*
8	6YR $\frac{6.7}{9.0}$	4	1Y $\frac{8.4}{5.0}$
7	8YR $\frac{7.2}{8.0}$	3	2Y $\frac{8.6}{4.6}$
6	1Y $\frac{8.2}{7.0}$	2	2Y $\frac{8.9}{2.5}$
5	10Y $\frac{8.2}{6.2}$	1	3Y $\frac{9.1}{1.5}$

Squares of these colors were mounted on stiff paper in series; an oblong hole 3 mm. wide was cut in the center of each to fit the upper incisors. Examination of the teeth was made in a dark room under a diffusing lamp such as is used for the examination of x-ray films. To obtain finer discrimination of shade, we examined the teeth through a Wratten (variant) filter no. 47. We measured only the upper incisors and tried as far as possible to measure the color in the middle third of the tooth.

The results of these measurements are shown in figure 4 where the age of the rat is plotted against the color of the teeth in units of chroma. These results show no variation with age in the color of the teeth of normal rats within the age limits observed. There is a marked discrepancy of color between the normal as compared with the abnormal teeth amounting to between 2 and 3 units of chroma. Perhaps more interesting still is the color of the teeth of the rats kept in the dark room on the 'McCollum' diet as compared with that of the radiated rats which were fed the same diet. At the age of 54 days (fig. 4) the dark room rats show very little color (3+ units) while at the same age the rats in room III have an appreciable amount of pigmentation (7+ units). The rats in rooms II and IV fall intermediate at 54 days, but later the curve approximates to that of room III. All the measurements at later ages of the dark room rats are at a lower level than those of the radiated animals. This result is extremely consistent and was apparent to us on naked eye observation (color photograph).

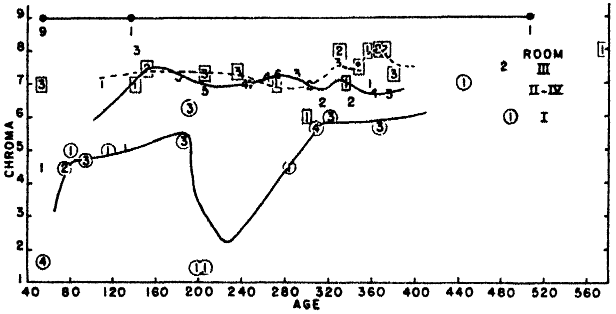

Fig. 4 Measurements of color of teeth. Upper line, rats on Sherman diet (normal); III, rats on 'McCollum' diet plus visible radiation; II and IV, rats on 'McCollum' diet plus near and far infra-red radiation; I, rats on 'McCollum' kept in darkness.

DISCUSSION OF RESULTS

Both McCollum ('25) and Smith and Lantz ('33) noted the change in color of the teeth and recognized it as an early symptom of fluorosis. The bleaching of the teeth, as seen in our dark room rats, occurred before any signs of deformity. In the radiated rats, even at 54 days, the color of the teeth is not normal. The possibility that the presence of fluorine in one of these diets, by providing an additional physiological stress, has intensified the effects of the lack of radiation in the affected animals, has been indicated in our studies on length ratios and color of the teeth.

Fig. 5 Tooth color in 54-day-old rats. Uniform colors in the upper and in the lower incisors of the rats fed the Sherman diet (left) are obtained in the dark (room I), in near infra-red radiation (room II) or in visible light (room III). Fluorosis ('McCollum' diet) produces a lack of pigmentation in both uppers and lowers which is most evident in the dark, intermediate in near infra-red radiation and more nearly normal in visible light. The shapes of the incisors are normal in every case at 54 days.

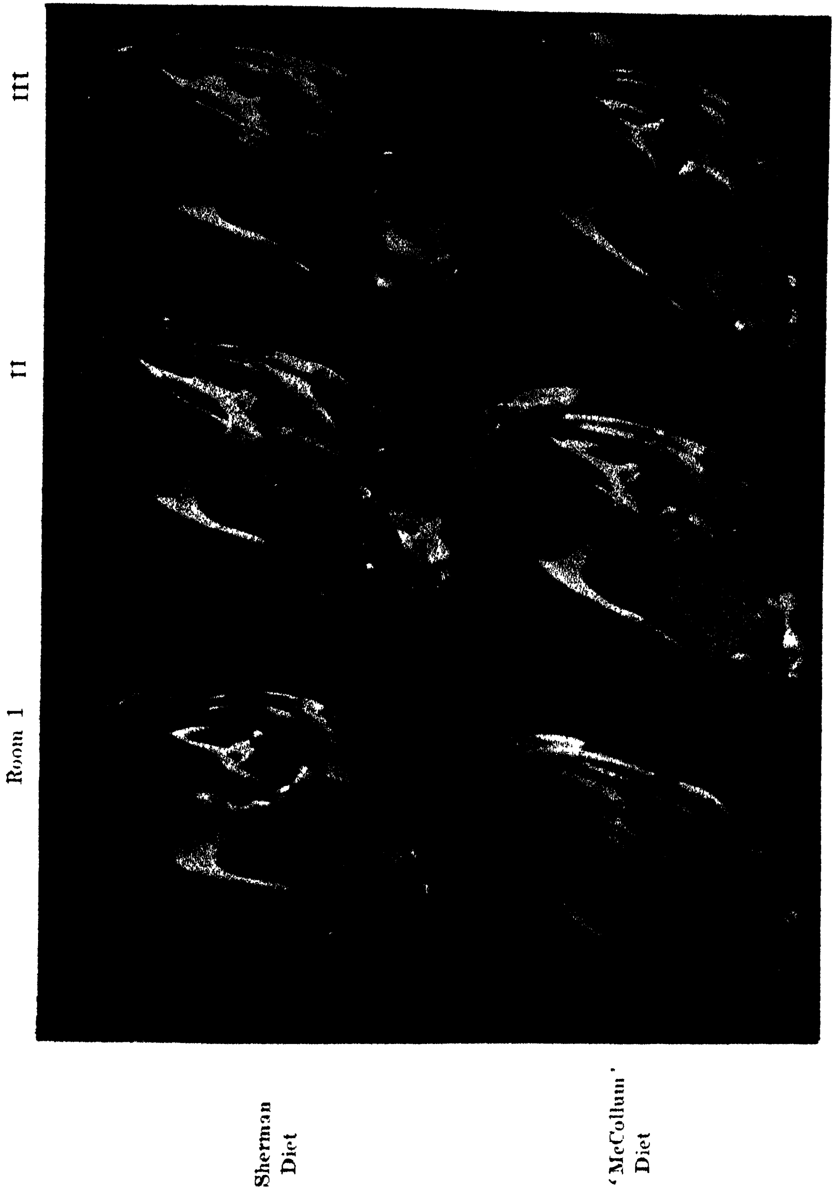

Figure 5

Little seems to be known about the physiology of pigment in the teeth of rats, nor as far as we can ascertain have the pigments present been identified. A suggestive fact in connection with light and color of teeth is the observation of Smith and Smith ('32) that in mottled enamel of human teeth, due to fluorine ingestion, the deposition of pigment is a secondary phenomenon and only appears on the lip line, or the part of the tooth exposed to light. Ellis and Maynard ('36) regard analysis for fluorine as a more sensitive test for slight degrees of fluorosis than pigment changes in teeth. It may be that in the absence of light, early pigment changes would be more apparent than in experiments conducted under ordinary laboratory conditions of light.

The source of contamination of the casein used in these experiments is unknown to us. We have no reason to suspect that such a contamination of commercial casein is of common occurrence. For though without any doubt it did occur in the casein used during the period of our experiment, yet we have recently (November, 1938) examined samples of casein, both commercial and purified, from several companies including the one from which we obtained our casein, and found them free of fluorine (on spectroscopic examination).

Our chief interest in writing up this work was to study the possible effects of radiation observed in animals fed the fluorine-contaminated diet.

The authors acknowledge with pleasure the assistance of the following persons: Dr. E. V. McCollum for reading the manuscript and for helpful criticism; Dr. L. T. Steadman for the spectrographic analyses; Dr. G. H. Ellis for the chemical analyses of the casein; Dr. W. D. Armstrong for the chemical analyses of the enamel and dentine samples; Dr. G. B. Van Schaak for mathematical study of the incisor geometry; Dr. B. J. Frey for measurements of eruption rate of the incisors, and Mr. J. W. Forrest for the Munsell values of the color comparator.

CONCLUSIONS

1. The occurrence of fluorosis in the teeth of rats due to a contamination with fluorine of commercial casein used in an otherwise standard diet, has been described.

2. Spectroscopic analysis of the casein showed approximately 0.1% fluorine.

3. Chemical analysis of the casein showed 0.2% fluorine.

4. Chemical anlysis for fluorine of the incisor teeth of the affected rats showed a concentration which was ten to twenty-five times the normal content.

5. Measurements of length of teeth showed a great increase of upper incisors as compared with normal.

6. Measurements of the color of the teeth showed marked diminution, as compared with normal, in all affected animals.

7. The eruption rate of the teeth of the affected rats was markedly slower than normal.

8. The measurements of length and color of the teeth gave some evidence that the presence of fluorine in the diet intensified the effects of lack of radiation in the affected animals.

LITERATURE CITED

ARMSTRONG, W. D. 1936 Micro-determination of fluorine. Proc. Soc. Exp. Biol. Med., vol. 34, p. 731.

BOWES, J. H., AND M. M. MURRAY 1935 XIV. The chemical composition of teeth. I. The estimation of fluorine and the fluorine content of normal teeth. Biochem. J., vol. 29, p. 102.

CHURCHILL, H. V. 1931 Occurrence of fluorides in some waters of the United States. Ind. Eng. Chem., vol. 23, p. 996.

ELLIS, G., AND L. A. MAYNARD 1936 Effect of low levels of fluorine intake on bones and teeth. Proc. Soc. Exp. Biol. Med., vol. 35, p. 12.

EVANS, H. M., AND K. S. BISHOP 1922 On the relation between fertility and nutrition I. The ovulation rhythm in the rat on a standard nutritional regime. J. Met. Res., vol. I, p. 319.

GASSMANN, TH. 1908 Chemische Untersuchungen der Zahne. Z. Physiologische Chemie, Bd. 55, S. 455.

MCCOLLUM, E. V., NINA SIMMONDS, J. E. BECKER AND R. W. BUNTING 1925 The effect of additions of fluorine to the diet of the rat on the quality of the teeth. J. Biol. Chem., vol. 63, p. 553.

MUÑOZ, JUAN M. 1936 El fluor de los huesos y dientes en la fluorosis. Revista de la Sociedad Argentina de Biologia, vol. 12, p. 50.

SANCHIS, J. M. 1934 Determination of fluorides in natural waters. Ind. Eng. Chem., Anal. Ed., vol. 6, p. 134.

SHERMAN, H. C., AND H. L. CAMPBELL 1924 Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet. J. Biol. Chem., vol. 60, p. 5.

SMITH, M. C., AND E. M. LANTZ 1933 Experimental production of mottled enamel. Tech. Bull. no. 45, Agr. Exp. Sta., Univ. of Arizona.

———— 1935 Further studies in mottled enamel. J. Am. Dent. Assoc., vol. 22, p. 817.

SMITH, H. V., AND MARGARET C. SMITH 1932 Mottled enamel in Arizona and its correlation with the concentration of fluorides in water supplies. Tech. Bull. no. 43, Agr. Exp. Sta., Univ. of Arizona.

WILLARD, H. H., AND O. B. WINTER 1933 Volumetric method for the determination of fluorine. Ind. Eng. Chem., Anal. Ed., vol. 5, p. 7.

THE EFFECT OF PROTEIN AND EXERCISE AT DIFFERENT AGES ON THE TOTAL ENERGY METABOLISM

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THREE FIGURES

(Received for publication October 27, 1938)

This investigation was undertaken to study over long periods of time and under normal conditions of living and activity the total or over-all metabolism of experimental animals. It is the total energy metabolism, not basal, which determines food requirement and presumably is related to endurance in the broader sense, to resistance to disease, and possibly to longevity of the individual.

The specific problem was to study the effect of protein and exercise with advancing age of the experimental animal on the total energy metabolism. The source of protein as well as the level were considered by incorporating in rations two widely used proteins at an 8% and a 20% level. The two proteins chosen were supplied by whole egg and whole milk, chosen because this department in previous studies by Sumner ('38) and Sumner and Murlin ('38) had established their true digestibility and biological values in both rat and man.

The interest in this work was augmented by the program of studies being pursued by McCay, Maynard and associates (McCay, Crowell and Maynard, '35; McCay, Maynard and Sperling, '38) on the influence of various nutritional factors on the length of life of the experimental animal. The problem of especial interest in these studies was the effect of protein and exercise on the total life span of rats (private communication).

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[1] The data in this paper are taken from a thesis presented in partial fulfillment of the requirement for the doctorate of philosophy, University of Rochester, June, 1938.

PLAN OF EXPERIMENT

The experimental subjects were ninety-six albino rats selected in groups of three, each group being matched as to sex, litter, age and weight. The experiment started shortly after the rats were weaned.

One-half of the rats or sixteen of the matched triplets were fed rations in which the protein was supplied by whole milk powder, while the other half of the animals received rations in which the protein was supplied by dried whole egg.

Within each triplet, two of the animals received a 20% protein ration and the third received a ration containing only 8% protein. One-half of the rats on the high protein ration were exercised daily to subdivide this group further.

The paired-feeding method was used as modified to include three animals in a group instead of two. The feed intake for all three was determined by the poorer eater of the two non-exercised animals. All diets from each protein were equicaloric. Those from milk and egg differed only slightly.

During the course of the experiment some difficulty was encountered in keeping all three animals of a triplet on the same food consumption and slight modifications were made at times to the rule. However, only one case occurred where the feed consumption was seriously out of balance and in this case the triplet was dropped before the end of the experiment.

The rations in which the protein was supplied by whole milk powder were composed as follows:

Cellu flour	4%
Sodium chloride	1%
Osborne & Mendel salt mixture	1 to 3%
Dried brewer's yeast	4 to 8%
Cod liver oil	1 to 2%
Whole milk powder[3]	16.65 to 71.38%
Cornstarch	15.62 to 48.74%
Crisco	0.00 to 16.61%

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[3] Klim.

The rations in which the protein was supplied by dried whole egg were composed as follows:

Cellu flour	4%
Sodium chloride	1%
Osborne & Mendel salt mixture	3%
Dried brewer's yeast	6 to 8%
Cod liver oil	1 to 2%
Dried whole egg[a]	9.26 to 38.53%
Cornstarch	42.30 to 56.56%
Crisco	3.67 to 17.40%

[a] Veritas brand, purchased from Jaburg Bros., New York. Not an imported product.

As enough food to last the entire experiment could not be prepared at one time, a new lot of each ration was prepared as needed. Certain rats on the egg rations became very poor eaters after the experiment had been in progress about 105 days, indicating that there possibly might not be an optimum amount of vitamin B complex present. Additional yeast was given aside from the rations at this time to all the rats on the egg rations and when the last lot of rations was mixed, the yeast content was increased from 6 to 8% to avoid extra weighings. At this time the yeast content of the milk rations also was increased from 4 to 8% to prevent a great difference in the yeast consumption between the rats on the two rations.

The rats were exercised daily in a wooden barrel held in a horizontal position and revolved by means of an electric motor. A window was constructed in one end of the barrel so that one could be certain the rats were running and not simply sliding around. A means was also provided to ventilate the barrel while a group of rats were being exercised. Exercise on calorimeter days always preceded the heat measurement by at least 3 hours.

The program of exercising was started during the third and fourth week and continued throughout the experiment. Only a moderate amount of exercise was imposed at the start but was increased as the experiment progressed. The rats were running a distance in the barrel at the end of the first month of exercising equivalent to 997 feet, and 25 days later a distance of 1661 feet. No further change was made until the experiment had been in progress 196 days, at which time the exercise was increased to 2325 feet daily. This amount of exercise was reasonably severe since it was equivalent to a steady run of 35 minutes.

After the experiment had been in progress approximately 6 months, the number of animals in each group was reduced from sixteen to twelve as the animals were much larger and a smaller number could, therefore, be used satisfactorily in the calorimeter. The reduction was made so as to leave in each group an equal distribution of sex of as uniform weight as possible. Obviously all three animals of a triplet were either retained or discarded.

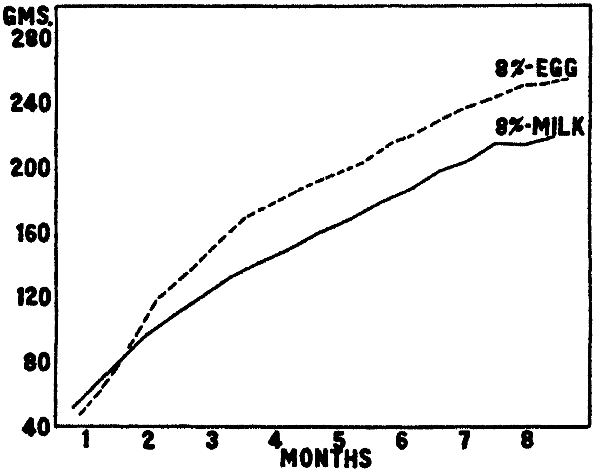

Fig. 1 Average growth of rats with the same energy intake but different source of protein.

The heat production was measured by means of a new automatic calorimeter which has been described recently by Barrows and Murlin ('38). A group of twelve to sixteen rats was placed in the calorimeter in small individual cages at one time and the heat production was measured continuously for 19 to 22 hours by both direct and indirect methods. Each rat had a day's food supply and water and was permitted to move around within the limits of the individual cage at will.

The apparatus gave theoretical values for heat as well as for the oxygen, carbon dioxide and R.Q. of alcohol. The data are omitted in the interest of economy of space.

RESULTS

The growth curves of the twelve rats from each of the six groups that were kept for the entire experiment are shown in figures 1 and 2. An equal distribution of sexes is represented in all the curves.

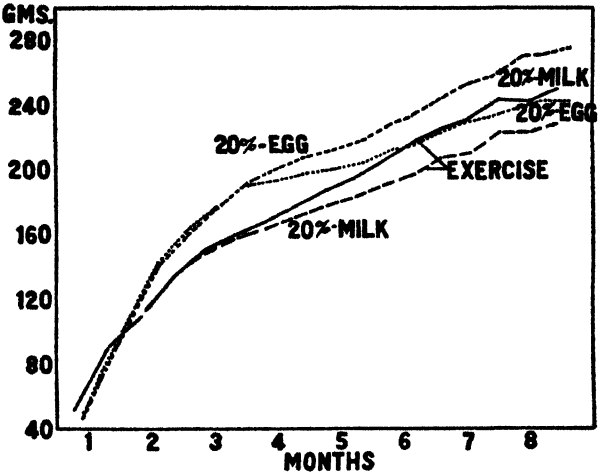

Fig. 2 Average growth of rats with the same energy intake but different source of protein.

A comparison of the growth promoting qualities of the proteins of two such important and widely used foods as egg and milk is of considerable practical value. The three groups of rats on the milk rations gained on the average 199, 168 and 176 gm. for the 20% protein, 8% protein and 20% protein and exercise groups respectively; while the gain on the egg rations was 229, 207 and 195 gm. for the 20% protein, 8% protein and 20% protein and exercise groups respectively. The gain of the group on the 20% egg protein ration of 229 gm. is a little high since one female rat of this group died before the end of the experiment. If the last weight taken be considered as the final weight for the rat that died, then the average gain of 229 gm. is 6 gm. high. The odds expressing the degree of significance of the gains in body weight, computed by the modification of Love ('24) of the method of 'Student' ('08) were as follows:

	Milk rations	*Egg rations*
Gain in weight of 20% protein over 8% protein	10,000: 1	195: 1
Gain in weight of 20% protein over 20% protein with exercise	3,332: 1	3,332: 1
Gain in weight of 20% protein with exercise over 8% protein	19: 1	.....
Gain in weight of 8% protein over 20% protein with exercise	.....	908: 1

The low protein group on the egg ration made a rather remarkable gain, approaching sufficiently near the high protein group that the odds (195: 1) do not indicate a difference of great significance. This was not true on the milk rations as the gain made by the high protein group was very significantly greater (odds 10,000 to 1) than by the low protein group. The superiority of egg protein over that of milk was best shown by comparison of the low egg protein with that of the high milk protein, the gain on the former being slightly greater than on the latter.

The inclusion of the two exercised groups affords a comparison not usually made in nutrition studies, namely to test the efficiency of the rations for producing work. The growth curves show that early in the study when little exercise was given the group on the egg ration gained weight faster than the group on the milk ration. This was to be expected, but when more exercise was given the difference was not so marked, and the growth curves for the two exercised groups tended to become closer as the experiment progressed. On the milk rations the low protein group gained less than the exercised group throughout, but on the egg rations the low protein group gained more than the exercised group, though only in the last 2 months. The difference was slight in the case of the groups receiving milk rations but quite significant at the end in the case of the groups receiving egg rations. From the foregoing comparisons there appears to have been a greater expenditure of energy by the group receiving the egg ration than by the group receiving the milk ration for producing an equal amount of work.

The greater gain made by the high protein groups, which were not exercised, was apparently due to a larger deposition of fat as judged by the amount of subcutaneous fat.

As the amount of feed given the rats on the milk rations was determined independently from the amount given those on the egg rations, it was not expected the total intake of feed for all six groups would be approximately the same at the end of the experiment, yet this proved to be the case. This coincidence is of considerable value in that all six groups can be compared on the basis of approximately equal energy intakes. The total nitrogen intake of the four groups on high protein rations also was very similar. This information is summarized in table 1.

TABLE 1

Total nitrogen and energy intake by groups for the entire experiment

Group	*Nitrogen gm*	*Energy Calories*
20% protein-milk	901.53	138,971.9
8% protein-milk	368.06	140,302.1
20% protein-milk with exercise	899.22	138,611.0
20% protein-egg	897.16	136,591.8
8% protein-egg	373.93	136,087.1
20% protein-egg with exercise	904.67	137,763.2

All of the egg rations produced greater growth than the corresponding milk rations notwithstanding that some less energy was consumed on the former. The significance of the results is increased by a difference of this nature. Errors which may be expected to occur in chemical analyses alone are sufficient to account for most of the differences in nitrogen and energy intakes between the various groups.

The heat production values presented in table 2 are the averages of the direct and indirect heat determinations for an equal number of periods equally distributed for each group. The lengths of the periods are 3 hours for period 1 and 16 hours for period 2; and in cases where there are periods 1, 2 and 3, the first two are of 3 hours duration each and the third of 16 hours duration. The surface area was computed by the use of Diack's ('30) formula.

There is an average difference between the direct and indirect heat measurement in eighty-one separate periods of 4.9%, with a standard deviation of 3.6 ± 0.19. The agreement between the direct and indirect heat measurement is quite as close with this apparatus as with the conventional type of calorimeter; besides the ease of operation is remarkably greater, as an operator must be present only a very small amount of the time. The deviation between the two methods of measuring the heat was not always in the same direction; the directly determined heat was higher than the indirect approximately as many times as it was lower.

The average total heat production for the experiment for all groups of animals with standard deviations is given in table 3. In figure 3, the heat production is plotted according to increasing age of the experimental animals.

As shown in figure 3, the heat production of all six groups of rats decreased with increasing age for about 5 to 6 months. A decrease in basal metabolism with increasing age is clearly established for most species of animals. As the basal constitutes such a large part of the total metabolism, the latter would be expected to follow generally the trend of basal metabolism.

The rise in total metabolism after a rather long period of decline was an unexpected finding. This increase occurred earlier in the two high protein groups on the egg rations than in any other groups. The high point on the curve of heat production for the sixth month for the 20% protein group from eggs cannot reasonably be attributed to a poor heat determination as there was good agreement between the two

TABLE 2

Summary of heat production determinations

GROUP	AGE OF RATS	PERIOD	HEAT PRODUCTION PER SQUARE METER OF SURFACE	GROUP	AGE OF RATS	PERIOD	HEAT PRODUCTION PER SQUARE METER OF SURFACE	GROUP	AGE OF RATS	PERIOD	HEAT PRODUCTION PER SQUARE METER OF SURFACE
20% protein from milk	59	1	44.4	20% protein from milk exercise	63	1	41.8	8% protein from eggs	63	1	49.4
20% protein from milk	59	2	45.6	20% protein from milk exercise	63	2	45.9	8% protein from eggs	63	2	50.3
20% protein from milk	143	1	41.9	20% protein from milk exercise	147	1	44.0	8% protein from eggs	149	1	37.2
20% protein from milk	143	2	44.2	20% protein from milk exercise	147	2	42.0	8% protein from eggs	149	2	41.2
20% protein from milk	171	1	41.3	20% protein from milk exercise	161	1	43.1	8% protein from eggs	163	1	36.8
20% protein from milk	171	2	43.7	20% protein from milk exercise	161	2	40.8	8% protein from eggs	163	2	39.8
20% protein from milk	199	1	39.0	20% protein from milk exercise	203	1	42.1	8% protein from eggs	205	1	37.9
20% protein from milk	199	2	39.2	20% protein from milk exercise	203	2	41.6	8% protein from eggs	205	2	35.8
20% protein from milk	199	3	42.7	20% protein from milk exercise	203	3	41.0	8% protein from eggs	205	3	38.7
20% protein from milk	248	1	43.2	20% protein from milk exercise	252	1	44.0	8% protein from eggs	254	1	39.1
20% protein from milk	248	2	44.1	20% protein from milk exercise	252	2	43.0	8% protein from eggs	254	2	38.2
20% protein from milk	248	3	46.6	20% protein from milk exercise	252	3	46.0	8% protein from eggs	254	3	43.3
8% protein from milk	61	1	42.8	20% protein from eggs	64	1	45.0	20% protein from eggs exercise	67	1	43.0
8% protein from milk	61	2	53.4	20% protein from eggs	64	2	45.9	20% protein from eggs exercise	67	2	47.8
8% protein from milk	145	1	41.6	20% protein from eggs	147	1	38.7	20% protein from eggs exercise	151	1	44.1
8% protein from milk	145	2	48.1	20% protein from eggs	147	2	38.6	20% protein from eggs exercise	151	2	39.7
8% protein from milk	158	1	38.3	20% protein from eggs	161	1	37.3	20% protein from eggs exercise	165	1	42.0
8% protein from milk	158	2	43.8	20% protein from eggs	161	2	39.3	20% protein from eggs exercise	165	2	37.5
8% protein from milk	201	1	37.8	20% protein from eggs	203	1	37.9	20% protein from eggs exercise	207	1	44.4
8% protein from milk	201	2	36.0	20% protein from eggs	203	2	37.2	20% protein from eggs exercise	207	2	41.7
8% protein from milk	201	3	43.9	20% protein from eggs	203	3	39.3	20% protein from eggs exercise	207	3	41.6
8% protein from milk	250	1	40.3	20% protein from eggs	252	1	39.2	20% protein from eggs exercise	256	1	44.2
8% protein from milk	250	2	44.9	20% protein from eggs	252	2	37.7	20% protein from eggs exercise	256	2	45.1
8% protein from milk	250	3	44.0	20% protein from eggs	252	3	41.7	20% protein from eggs exercise	256	3	43.6

methods of determining the heat production. An unusual amount of activity must have occurred during this calorimeter determination to have increased the heat production by such an amount.

The increased heat production which occurred in the latter part of the experiment was apparently caused by a number of factors each contributing a part. Some of the factors which may reasonably be supposed to have influenced the heat production are the following:

The growth impulse in the rat is tremendous. A good example of this is the ability of the rat to grow considerably after 2 or 3 years of retardation (McCay, Crowell and Maynard, '35). As the paired-feeding method does not permit the maximum growth rate, the rat can reasonably be expected to be less wasteful of energy until the growth impulse is satisfied. Thereafter more energy remains unclaimed for retention which would appear as dynamic effect.

TABLE 3

Average heat production[1] *for equal number of determination on each group*

GROUP	MILK RATIONS		EGG RATIONS	
	Average age	Heat production	Average age	Heat production
	days	*Cal./sq.m /hr*	*days*	*Cal./sq m./hr.*
20% protein	164	43.0, $\sigma = 2.2$	165	39.8, $\sigma = 2.8$
8% protein	163	42.9, $\sigma = 3.3$	167	40.6, $\sigma = 3.8$
20% protein + exercise	165	42.9, $\sigma = 1.7$	169	42.9, $\sigma = 2.5$

[1] Values shown are based on the same number of long and short periods, equally distributed as to age.

Another circumstance to help explain the increased heat production as found would be an increase in activity. There is much less inclination and need for a mature animal to sleep than one that is young and growing rapidly. The total activity may be roughly proportional to the amount of time spent awake.

The activity may also have been increased by thwarted sexual impulse which must be strong in rats from 5 months upward especially as both sexes were in small individual cages in the calorimeter at one time.

Another point shown by the curve of heat production is that the low protein groups on both the egg and milk rations had a higher heat production at the start of the experiment, lasting to beyond 4 months on the milk ration, than the high protein groups. This confirms the work of Forbes, Swift, Black and Kahlenberg ('35) in which a higher heat production was found on a 10% protein ration than any protein level up to 25% in experiments of 10 weeks of duration. But in the present investigation the lowest heat productions also were obtained later in the experiment, when the animals were older, with the same low protein groups.

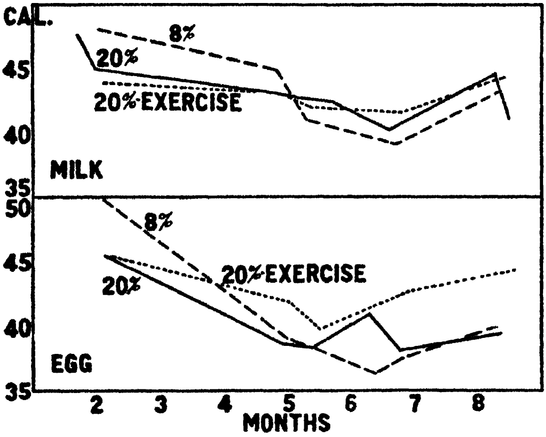

Fig. 3 Total heat production with increasing age as affected by differences in source and amount of protein intake and by exercise. All heat measurements that were made are included in this figure.

There was less variation as regards heat production between the two exercised groups than between any other two groups. As these animals did not get all they could eat and were exercised uniformly each day, the total activity would not be expected to vary so much throughout the experiment.

The average heat production of an equal number of periods equally distributed for the entire experiment was essentially the same for the three groups on the milk rations as shown in table 3. The exercised group on the egg ration had a higher average heat production than the two other groups on the egg rations that were not exercised. This difference was accumulated throughout most of the experiment, as the heat production curve (fig. 3) was highest for the exercised group at all times except early in the experiment when little exercise was given. The exercised group converted less feed energy into body gain than did the other two groups on the egg rations.

On the basis of the average heat production values for the experiment, the level of protein intake did not influence the heat production. The source of protein did affect slightly the heat, as there was a lower production on the egg rations than on the milk rations on both the high and low intake, except in groups of the experimental animals which were exercised. When protein is retained for growth of muscle or other tissue, it is withdrawn from dynamic effect. Milk does not produce tissue so well, and it appears to cause a greater specific dynamic effect with consequent higher heat production. However, these differences prove not to be statistically significant. Thus in table 3 the means for 20% milk and egg are 42.4 and 39.8 Cal./square meter/hr. respectively. This difference between means of 2.6 Cal. is not greater than the square root of the sum of the squares of the two standard errors of the means. Significance requires that the difference be two times (Davenport and Ekas, '36) this square root.

SUMMARY

The effects of the proteins of whole milk powder and dried whole egg, incorporated in equicaloric rations at both a high and a low level (8 and 20%), were studied on the total energy metabolism of rats from weanling to 8½ months of age with a direct-indirect automatic calorimeter. The effects of a mild amount of exercise were also studied in one-half of the animals on the high protein ration. Feeding was by the paired method as modified to include three animals on the same protein in a group.

A marked superiority of the protein of egg over milk for producing growth was shown when the energy intake was the same.

The total heat production of all six groups of animals, expressed on a surface area basis, decreased rather decidedly with increasing age during the first 5 to 6 months of the experiment. After this an increase in heat production was found in all groups which continued at an increasing rate until the end of the experiment. Possible reasons for this are proposed.

The average total metabolism for the entire experiment was essentially the same for the three groups on the milk rations. It was lower for the two non-exercised groups on the egg rations than for the exercised groups.

The level of protein did not influence the average total heat production.

LITERATURE CITED

BARROWS, W. M., JR., AND J. R. MURLIN 1938 New automatic calorimeter for the continued measurement of heat production over long periods of time. Am. Philos. Soc., Proc., vol. 78, pp. 483–512.

DAVENPORT, C. B., AND M. P. EKAS 1936 Statistical Methods in Biology etc. Wiley & Sons, New York.

DIACK, S. L. 1930 The determination of the surface area of the white rat. J. Nutrition, vol. 3, pp. 289–296.

FORBES, E. B., R. W. SWIFT, ALEX BLACK AND O. J. KAHLENBERG 1935 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. III. The effects of the plane of protein intake. J. Nutrition, vol. 10, pp. 461–479.

LOVE, H. H. 1924 A modification of Student's table for use in interpreting experimental results. J. Am. Soc. Agron., vol. 16, pp. 68–73.

MCCAY, C. M., M. F. CROWELL AND L. A. MAYNARD 1935 The effect of retarded growth upon the length of life span and upon the ultimate body size. J. Nutrition, vol. 10, pp. 63–79.

MCCAY, C. M., L. A. MAYNARD AND GLADYS SPERLING 1938 The retardation of senescence. J. Nutrition, vol. 15, p. 12, supplement.

'STUDENT' 1908 The probable error of the mean. Biometrika, vol. 6, pp. 1–25.

SUMNER, EMMA E. 1938 The biological value of milk and egg protein in young and mature rats. J. Nutrition, vol. 16, pp. 129–141.

SUMNER, EMMA E., AND J. R. MURLIN 1938 The biological value of milk and egg protein in human subjects. J. Nutrition, vol. 16, pp. 141–153.

THE EFFECT OF PROTEIN AND EXERCISE AT DIFFERENT AGES ON THE BASAL METABOLISM[1]

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The investigation to be discussed is a part of a previous study of the effect of protein and exercise on the total energy metabolism reported in this issue of this Journal (Black and Murlin, '39).

In this study equicaloric rations containing 8 and 20% protein from both whole egg and whole milk were fed to rats from weanling to 8½ months of age. The rats on the high protein rations were divided into two groups, one group receiving a moderate amount of exercise daily. The three groups of animals receiving either source of protein were selected in triplets. Each animal of a triplet was of the same sex, litter, age and weight and was given the same amount of food.

During the seventh to the eighth month of the experiment, basal metabolism determinations were made on a representative number of animals from each group.

The results of the total energy metabolism investigations together with a full description of the experimental procedure followed in the treatment of the animals is given in the previous paper.

The study of the basal metabolism of rats much older than those just described was made possible through the cooperation of the Laboratory of Animal Nutrition of Cornell University. The investigation dealing with total energy metabolism was planned so that comparisons in the energy metabolism of the animals in this experiment could be made with those in the study of the effect of protein and exercise on the length of life of rats which was being conducted by McCay and Maynard at Cornell. Since these workers were not equipped to study energy metabolism, they very kindly relinquished that phase of the investigation to this laboratory.

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[1] Part of a thesis presented in partial fulfillment of the requirement for the doctorate of philosophy, University of Rochester, June, 1938.

By transporting the metabolism apparatus to the Cornell Laboratory, basal metabolism determinations were made on a number of very old rats which had received comparable treatment to those used in this department.

The apparatus used for basal metabolism determinations was of the Haldane principle; moisture and carbon dioxide production and the oxygen consumption were determined gravimetrically. The particular form of apparatus used was constructed similarly to the one described by Forbes, Kriss and Miller ('34), with a few minor modifications.

All measurements were made with the respiration chamber maintained at a temperature between 28°C. and 29°C. The length of the experimental periods was from 6 to 7 hours. The carbon dioxide production for the first hour, and occasionally the second hour, was always excluded from the calculation of the heat production because of activity of the animal; however these hours were not excluded from the calculation of the respiratory quotient. The heat production was computed from the average respiratory quotient and the hourly carbon dioxide production.

The basal heat productions of the rats selected from the experiment in this department are shown in table 1. These values are expressed as calories per square meter of body surface per hour. Surface area was estimated by the use of Diack's ('30) formula. The plan followed in the selection of rats from each group for basal metabolism determinations was to use matched animals, and therefore an equal distributions of sexes was represented.

TABLE 1

Basal heat production, rats at Rochester

PROTEIN SUPPLIED BY MILK					PROTEIN SUPPLIED BY EGGS				
Rat	Sex	Basal heat per hour	Surface area	Basal heat per square meter per hour	Rat	Sex	Basal heat per hour	Surface area	Basal heat per square meter per hour
		Cal.	*sq cm.*	*Cal.*			*Cal.*	*sq cm*	*Cal.*
		20% protein ration					20% protein ration		
1	M	1.073	321	33.4	58	F	0.777	263	29.5
4	F	0.838	251	33.4	61	F	0.842	260	32.4
13	M	1.131	321	35.2	64	M	1.105	361	30.6
19	M	1.066	335	31.8	67	F	0.803	273	29.4
22	M	1.348	334	40.4	73	M	1.125	375	30.0
25	F	0.728	245	29.7	79	M	1.014	323	31.4
34	F	0.756	255	29.6	85	M	1.060	334	31.7
37	M	0.913	302	30.2	Average		0.961	312	30.7
Average		0.982	296	33.2					
		8% protein ration					8% protein ration		
2	M	0.872	296	29.5	59	F	0.709	254	27.9
5	F	0.755	235	32.1	62	F	0.809	255	31.7
14	M	0.942	310	30.4	65	M	1.137	352	32.3
20	M	0.930	304	30.6	68	F	0.790	265	29.8
23	M	1.001	303	33.0	74	M	1.239	360	34.4
26	F	0.717	225	31.9	80	M	0.973	306	31.8
35	F	0.655	228	30.0	86	M	1.092	320	34.1
Average		0.845	272	31.1	Average		0.964	302	31.9
		20% protein ration and exercise					20% protein ration and exercise		
3	M	1.220	312	39.1	60	F	0.830	250	33.2
6	F	0.850	237	35.9	63	F	0.933	255	36.6
15	M	1.055	306	34.5	66	M	1.226	341	36.0
21	M	1.099	293	37.5	69	F	0.821	272	30.2
24	M	0.979	295	33.2	81	M	1.080	292	37.0
27	F	0.708	230	30.8	87	M	1.068	304	35.1
36	F	0.837	228	36.7	Average		0.993	286	34.7
Average		0.964	272	35.4					

In table 2 the basal heat productions of the rat studied at Cornell are given as well as the age, treatment and general condition of the animals. This experiment was designed to study the effect of the proteins, casein and liver, fed at a high and low level, with and without exercise, on the total life span. All the animals in this experiment were males. The food consumption was not determined, but the animals were in general, showing a slight decrease in weight. Some loss in weight may be expected as a natural consequence in rats of this age.

The rations fed in this experiment were composed of certain constant constituents which were as follows: sucrose 10%, cellulose 2%, butter 5%, yeast (partly irradiated) 5%, salt mixture 3 to 4% and carotene at the rate of 1 mg. per kilogram of ration. In addition to these constant constituents the remainder of the four rations was composed as follows: high protein ration from casein: cooked starch 34%, casein 29%; lactalbumin 4% and lard 7%; high protein ration from liver: cooked starch 34% and dry liver 41%; low protein ration from casein: cooked starch 65%, casein 4%, lactalbumin 3% and lard 2%; low protein ration from liver: cooked starch 65% and dried liver 10%.

The high and low protein rations from casein contained approximately 27 and 7% protein respectively while the corresponding rations from liver contained 28 and 9% protein.

There was observed a wide fluctuation in the basal metabolism of the old rats that cannot be attributed to any external conditions. The animals were less active on the whole than the younger ones were during the time they were in the respiration chamber. One animal, K205, was not active but was in a state of high muscular tension as noted by one leg quivering at times. A high metabolism could therefore be expected under these circumstances.

Four rats in this group breathed with apparent difficulty; however, this peculiarity of breathing was manifest whether in the respiration chamber or the regular cage. The respirations were rather slow, very deep and seemed to require extra

TABLE 2

Basal heat production, male rats at Cornell

RAT	WEIGHT	PROTEIN FED	PROTEIN LEVEL	AGE	BASAL HEAT/SQ. METER/HR. (DIACK)	CONDITION OF ANIMALS
				days	*Cal.*	
Rats exercised						
K 649	298	Casein	High	673	41.5	Good
K 767	203	Liver	High	641	43.4	Very thin, died in run
K 229	264	Casein	High	859	42.4	Good
K 652	301	Casein	High	676	38.0	Fair, very thin
K 827	323	Casein	High	636	39.2	Good
K 396	302	Liver	High	712	38.1	Fair
K 778	307	Liver	High	703	31.2	Good
K 816	307	Liver	High	700	38.7	Good
K 771	356	Liver	Low	636	32.9	Lost most of hair from stomach
K 41	321	Casein	Low	871	36.1	Good, some hair lost from stomach
K 831	333	Casein	Low	646	31.0	Good
K 205	342	Liver	Low	869	42.7	Fair, some hair lost from stomach, tense
K 224	333	Liver	Low	854	38.2	Hair very thin
K 504	411	Liver	Low	720	33.1	Good
K 517	359	Liver	Low	707	41.7	Good
K 819	357	Liver	Low	649	32.4	Fine
Average				722	37.5	
Rats not exercised						
K 391	328	Liver	High	708	46.4	Good
K 579	286	Casein	High	714	41.9	Fair, breathes hard
K 413	271	Liver	High	717	40.9	Fair, breathes hard
K 522	301	Liver	High	715	31.0	Fine
K 531	299	Liver	High	717	29.6	Fine
K 691	327	Liver	High	682	38.5	Very good
K 814	282	Liver	High	645	41.1	Good, breathes slightly hard
K 480	276	Casein	Low	713	40.4	Not very good, breathes hard
K 550	338	Casein	Low	713	36.1	Good
K 646	353	Casein	Low	680	30.5	Fine, reasonably fat
K 829	299	Liver	Low	641	30.3	Good
Average				695	37.0	

effort on the part of the animal. As expected, the metabolic rate of these rats was found to be high, but no higher than in others that did not have this peculiarity in breathing.[2]

Basal metabolism determinations were made on all the rats in this experiment that were alive at the time and which did not show pathological conditions or from natural reasons appeared near death. It was not anticipated that rat K767 was so near death, or it would not have been used. The chief interest was in basal metabolism of old age under as nearly normal conditions as possible without contamination with obvious pathological conditions.

RESULTS WITH RATS AT ROCHESTER

The high protein group on the milk rations produced on the average slightly more heat than the low protein group indicating that possibly the basal metabolism was lowered by a low protein ration. The odds are not significant (16:1) in this case as there are so few animals. The exercised group on the milk rations had the highest average heat production of any group on the milk rations, but again the odds do not indicate that this difference is significantly greater than the non-exercised high protein group. They do indicate a slight significance (odds 87:1) over the non-exercised low protein group.

The two non-exercised groups on the egg rations had nearly the same basal heat production, but the average for the low protein group was higher than for the high protein group in this case. The exercised group again had the highest basal heat production of any group on the egg ration as was the case on the milk rations. The odds that the higher basal metabolism of the exercised group (than that of the 20% protein non-exercised group), was not due to chance alone, are 666:1. The odds are 158:1 that the heat production of the exercised group was really greater than that of the low protein group.

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[2] Since this paper went to press Dr. McCay has discovered that his old rats are quite subject to a low-grade pneumonia.

If the corresponding groups are combined by not considering the source of protein, the highest basal heat production occurred in the high protein group receiving exercise and the lowest in the low protein group. The high protein group which was not being exercised produced slightly more heat on the average than the low protein group. The magnitude of the difference between the exercised group and the low protein group is expressed by odds of 3332:1, and the difference between the exercised group and the high protein group by odds of 103:1.

A number of differences found in the total metabolism have also been found to occur in the basal metabolism. The average total metabolism was the same for all three groups on the milk rations. While the basal metabolism for these groups was not the same, there were no differences of especial significance. The basal metabolism of the two non-exercised groups on the egg rations was lower on the average than the two non-exercised groups on the milk rations which was also true for the total metabolism. The total metabolism of the exercised group on the egg rations was considerably higher at the end of the experiment than the two other groups on the egg rations; also the basal metabolism was higher.

The explanation for the higher basal metabolism in the exercised animals may have been due to a higher degree of muscular tonus, less subcutaneous fat, more muscular tissue or other factors.

The feed intake of the exercised animals was limited by animals that were not being exercised, and this limitation was sufficient to keep the body weight well below the group that was not exercised. It is well known that undernutrition lowers the metabolism, but in spite of any effect this factor may have had on the basal metabolism of the exercised rats, the result was a higher metabolism. This lends further support to the significance of exercise increasing the basal metabolism.

The difference between the average total metabolism during the final 2½ months of the experiment and the basal metabolism was 26%, which is called supermetabolism. This difference includes all six groups of animals. The largest supermetabolism, 32%, occurred in the low protein group on the milk ration.

The generally accepted idea that the basal heat production of female rats is lower than that of males is upheld in this work, as the average of the twenty-four male rats is 804 and of the eighteen female rats is 760 Calories per square meter of body surface per 24 hours.

RESULTS WITH RATS AT CORNELL

Exercising of the rats in this experiment continued throughout the life of the animals until old age and was terminated approximately 5 months before the basal metabolism measurements were made. Under these conditions the exercise had not influenced the metabolism. If the prolonged program of work imposed on these animals speeded up the vital-functions of the body and, therefore, the basal metabolism and total energy expenditure, its effect was not permanent. The average of the eight rats on high protein diet which had been exercised was 39.1 Cal./sq.m./hr., while the average of the six on high protein which had not been exercised was 38.5 Cal. Also the basal metabolism of the low protein groups was very similar.

The basal heat production of the fifteen rats on the high protein intake was 38.8 Calories per square meter of body surface per hour, while it was only 35.5 Calories per hour in the twelve rats on the low protein intake. A difference of this magnitude is probably significant. In the experiment with the rats at Rochester where identical amounts of equicaloric rations of high and low protein content were fed, the basal metabolism was not altered.

The metabolism of one rat (K767) that died in the respiration chamber, was high immediately preceding death. There was also a reasonably large premortal rise.

In this experiment, the heat production was calculated by three different methods, Diack's formula, Lee's formula and per unit of body weight. The comparative results were unaffected by the method of calculation.

In comparing the basal metabolism of the young and old rats, a comparison of animals of the same weight has a distinct advantage since it eliminates the possibility of errors which may be involved in calculating the heat production to a comparable basis. In these experiments, it was possible to select eleven of the 7- to 8-month-old rats of almost exactly the same weight as eleven of the 700-day-old rats for such a comparison. The basal metabolism of the young rats was 814 Calories and of the 700-day-old rats was 924 Calories per square meter of body surface per 24 hours. This was certainly not due to activity as the older rats appeared quieter than the younger ones. The two methods of feeding cannot be wholly reconciled as being without some influence on the metabolism. It seems reasonable to think that some of the difference noted in the basal metabolism must have been due to age. There may be good reasons for old rats to show a higher basal metabolism than younger ones. The size of the organs, the amount of subcutaneous fat and the degree of relaxation are some of the factors which may be responsible for the difference in metabolism.

SUMMARY

The basal metabolism of rats 7 to 8 months of age was studied and compared with the total metabolism of these same animals and also with the basal metabolism of other rats 700 days of age. In the investigation involving the younger animals, the effects on metabolism of the proteins of whole milk and whole egg fed at a high and low level and of exercise were observed. With the older rats, the influence of the level of protein intake, exercise and no exercise, was studied on the length of life of the animal.

In the study involving the younger rats, the basal heat production was the lowest on the average in the low protein group, somewhat higher in the high protein group and highest in the high protein group that was being exercised. The difference in heat production between any two groups is not great except in the case of the low protein group and the high protein group receiving exercise, in which case the difference is quite significant.

The supermetabolism, or the difference between the total metabolism and the basal metabolism, was 26%.

With the old rats, a high protein intake was associated with a higher basal metabolism.

In comparisons between animals of equal weights, a higher basal metabolism was found in the older animals.

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LITERATURE CITED

BLACK, ALEX, AND J. R. MURLIN 1939 The effect of protein and exercise at different ages on the total energy metabolism. J. Nutrition, vol. 17, pp. 347–359.

DIACK, S. L. 1930 The determination of the surface area of the white rat. J. Nutrition, vol. 3, pp. 289–296.

FORBES, E. B., M. KRISS AND R. C. MILLER 1934 The energy metabolism of the albino rat in relation to the plane of nutrition. J. Nutrition, vol. 8, pp. 535–552.

THE PREVENTIVE EFFECT OF WHEAT GERM OILS AND OF α-TOCOPHEROL IN NUTRITIONAL MUSCULAR DYSTROPHY OF YOUNG RATS[1]

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Rats on certain vitamin E-low diets develop nutritional muscular dystrophy. The paralytic symptoms of this disease were observed for the first time in the laboratory of Evans and Burr ('28) in the young of female rats that had been given the minimum amount of vitamin E to produce a litter. The disorder appears on or about the twentieth day of life and is characterized by severe degeneration of the skeletal muscles (Olcott, '38). Symptoms of muscle weakness have been reported in older animals maintained on vitamin E-low diets from the time of weaning (Ringsted, '35; Burr, Brown and Moseley, '37; Einarsten and Ringsted, '38; Evans, Emerson and Telford, '38; Knowlton and Hines, '38). Several of these authors have also described slight lesions in the muscles.

Evans and Burr ('28) showed that the paralysis was prevented by the administration to either mother or young during the period of lactation, of wheat germ oil or vitamin E concentrates. The protective effect of wheat germ oil and concentrates of vitamin E was confirmed by Morelle ('31), Mason ('33), Olcott and Mattill ('34) and Ringsted ('35). However, in similar experiments with diets of natural foods in which

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the anti-sterility factor had been inactivated by treatment with ethereal ferric chloride (Waddell and Steenbock, '28), young rats remained normal (Goettsch and Pappenheimer, '31).

The present paper concerns the role of vitamin E in muscle dystrophy of the young rat. A comparison has been made of the anti-sterility potency and the muscle dystrophy preventing effect of wheat germ oil, of oil of treated wheat germ in which the vitamin E has been inactivated by ferric chloride treatment, and of α-tocopherol (Evans, Emerson and Emerson, '36).

EXPERIMENTAL

Diets and supplements. The diets, similar to those used by Evans and Burr ('28), had the following composition:

	Diet I %	*Diet II* %
Casein (Merck's technical)	29.1	26.6
Cornstarch	36.3	33.3
Lard	20.0	18.3
Yeast, bakers' dried	9.0	16.6
Salt mixture (Hawk and Oser, '31)	3.6	3.4
Cod liver oil (Mead Johnson)	2.0	1.8

Diet I is low in vitamin E for, in all female rats (thirty-four) reared and bred upon it, the first gestations resulted in resorptions. Diet II containing more yeast was used during the lactation period.

Wheat germ oil. Wheat germ was extracted in a continuous extraction apparatus with petroleum ether which had been purified by treatment with concentrated sulfuric acid for several weeks, followed by washing and distillation. The oil was used within a month of preparation.

Oil of treated wheat germ. Ten grams of ferric chloride, U. S. P. lump, was taken up in about 500 cc. ethyl ether and a little water and the solution poured over 1 kg. of wheat germ. The mass was shaken in a closed container, allowed to stand over night and extracted with ether.

α-Tocopherol. This material was kindly presented to us by Merck and Co. through the curtesy of Dr. Randolph T. Major.

Method of inducing muscular dystrophy in rats. Young female rats were reared on diet I from the time of weaning and bred at 90 days of age according to the technic of Evans and Burr ('27). In order to supply sufficient vitamin E to produce a litter, 1 gm. of wheat germ oil was given by mouth during the first 5 days of gestation. Although the feeding of 550 mg. of wheat germ oil to rats on vitamin E-low diets results in the birth of living young (Evans and Burr, '27), 1 gm. was fed in these experiments in the hope of obtaining more young rats that were viable.

Under these conditions, reproduction compared favorably with that found in normal stock rats. Fifty gestations resulted in forty-seven litters and three resorptions. The litters averaged 5.9 rats, since a total of 275 young were born. Thirty-three (12%) were born dead. The average birth weight of those born alive was 4.8 gm.

All of the young were kept for lactation during which period the diet of the mother was changed to diet II. During the first 10 days of lactation there was a mortality in the young, from unknown causes, of 25%. At the end of the period, from the nineteenth to the twenty-third day, symptoms of paralysis such as described by Evans and Burr ('28) appeared in many of the remaining rats. Among 143 young, 130 (90%) developed the disease and forty-six (35%) of those presenting symptoms died.

On the twenty-fifth day of lactation all of the young were killed. Autopsies were performed on every rat in the litter and representative muscle specimens preserved for histological examination.

Pathology. We are indebted to Dr. A. M. Pappenheimer for his study of the pathology of the muscle which will appear in detail elsewhere. The findings confirm those of Olcott ('38) in all essentials. The changes are strictly limited to the voluntary muscles.

Muscle creatine. In degenerated muscle of the rat, there is a striking loss of muscle creatine comparable to that found in nutritional muscular dystrophy of the rabbit (Goettsch and

Brown, '32; Morgulis and Spencer, '36) and the guinea pig (Madsen, '36). Creatine was determined by the method of Ross, Helmer and Chanutin ('27). The mean of twenty-eight creatine determinations in normal muscle of 25-day-old rats was 434.2 ± 4.4 mg. creatine per 100 gm. of fresh muscle; the standard deviation was ±34.2 ± 3.1 and the coefficient of variation 7.9. Normal muscle of adult rats had the same creatine content. During the first 21 days after birth, the muscle creatine in young rats increased from 140 mg. to the adult level. At 15 days of age, the muscle creatine averaged 300 mg.

In rats with severe muscular dystrophy, the muscle creatine was reduced to as low concentrations as 20 to 30 mg. per 100 gm. fresh tissue (table 1). The decrease corresponded roughly to the duration of the symptoms and to the amount of degeneration in the muscle. During recovery, when the muscle fibers were regenerating, the muscle creatine increased to the normal amount.

Test for the muscle dystrophy preventing factor. In testing for the muscle dystrophy preventing factor, some of the young of each litter were kept as controls and were not given any supplementary feeding. The remaining rats were fed the material to be tested. It was given by mouth daily from the tenth to the twenty-fifth day of lactation (Morelle, '31).

Table 1 illustrates not only the variation in extent of muscle lesions among those rats of a litter which had been given no supplementary feeding, but also the prevention of muscular dystrophy in those which had been fed small amounts of active preparations.

A total of forty-seven litters was used for muscle dystrophy preventing tests. Among these, there were forty litters in which all of the control rats presented degenerated muscles; five litters in which some of the controls were affected and some were normal; and only two litters in which none of the control rats developed muscular dystrophy.

TABLE 1

Showing the extent of degeneration and creatine content of muscles in normal young and those with nutritional muscular dystrophy

NUMBER OF LITTER	NUMBER OF YOUNG RAT	AGE IN DAYS	DOSAGE DURING LACTATION	SYMPTOMS MUSCLE DYSTROPHY		EXTENT OF MUSCLE LESIONS		MILLIGRAMS CREATINE PER 100 GM. FRESH MUSCLE
				Extent	Duration	Gross	Micro-scopic	
21–3	1	21	None	++++	2 days	++++	++++	70
	2	21	None	+++	2 days	+++	++++	165
	3	25	160 mg. W.G.O.	—		—	—	425
	4	25	160 mg. W.G.O.	—		—	—	430
36–3	1	24	None	++++	1 day	++	++++	150
	2	24	None	++++	1 day	++	++	290
	3	25	300 mg. T.W.G.O.	++	4 days	++	++++	220
	4	25	300 mg. T.W.G.O.	—		+	+++	385
77	1	25	None	—		—	—	405
	2	25	None	++	4 days recovered	+	++ regen.	300
	3	25	None	++	4 days recovered	+	+++ regen.	385
76	1	21	None	++++	2 days	+++	++++	60
	2	21	None	++++	2 days	+++	++++	135
	3	21	None	++	2 days	++	+++	380
35	1	23	None	++++	2 days	+++	+++	115
	2	23	None	++++	4 days	++++	+++	130
	3	25	300 mg. T.W.G.O.	—		—	regen.	400
	4	25	300 mg. T.W.G.O.	—		—	—	455
	5	25	300 mg. W.G.O.	—		—	—	435
	6	25	300 mg. W. G. O.	—		—	—	455
37	1	19	None	++++	1 day	+	++	205
	2	21	None	++++	2 days	++++	++++	20–30
	3	24	5 mg. α-tocopherol	—		—	—	465
65	1	25	None	+++	2 days recovered	++	+++ regen.	340
4	1	25	None	+++	6 days	++	+++	295
	2	25	None	++	5 days recovered	+	regen.	415
	3	25	None	—		—	—	405
8–2	1	25	None	+++	3 days recovered	++	++ regen.	350

Rats that were protected from muscle dystrophy grew as well as rats upon the stock diet. Their average body weight at 21 days was 40 gm.

It was possible to use several litters from the same female rat. After lactation, the rat was given diet I, allowed to rest for about 2 weeks, bred, and given 1 gm. of wheat germ oil. The resulting litter was used for another muscle dystrophy preventing test. In some instances, three litters were used with consistently good results.

Results of feeding preparations. Wheat germ oil, oil of treated wheat germ in which vitamin E had been inactivated by ethereal ferris chloride and α-tocopherol were fed under the specified conditions. Preliminary experiments indicated that all three preparations prevented muscular dystrophy in young rats. The final results are given in table 2.

Wheat germ oil. Ten rats in four litters were protected from muscle dystrophy when 2 drops of wheat germ oil (a total amount of 600 mg.) were given daily from the tenth to the twenty-fifth day of lactation. When the amount fed was decreased to 1 drop of wheat germ oil daily (a total of 300 mg.), fifteen out of sixteen rats in seven litters were protected. These results are in accord with those of Evans and Burr ('28).

Oil of treated wheat germ. The oil extracted from wheat germ that had been treated with ferric chloride also prevented muscle dystrophy, but a larger amount was necessary. Twelve rats in six litters, receiving 2 drops daily (a total of 600 mg.), were protected against the disease. When 1 drop daily (a total of 300 mg.) was given, only six of twelve rats in five litters remained normal.

Since 300 mg. of oil of treated wheat germ was not so active as 300 mg. of untreated wheat germ oil in protecting the rats against degenerated muscle, it is apparent that ferric chloride treatment had inactivated some of the muscle dystrophy preventing factor.

This oil extracted from treated wheat germ was tested for vitamin E potency in the usual manner. It failed to facilitate the production of living young in twenty rats that had had resorptions on diet I. The following amounts were given: 1 gm. per gestation to seven rats; 2 gm. to three rats; 12 gm. to four rats, one of which had a litter of two dead young; and 20 gm. to six rats. That these rats retained their normal response to vitamin E was proved by the fact that litters

TABLE 2

Showing the protective effect of wheat germ oil, oil of treated wheat germ and α-tocopherol in nutritional muscular dystrophy in the young rat

FEMALE RAT	NO SUPPLEMENT		WITH SUPPLEMENT		DOSE
	Number of rats	Number with muscular dystrophy	Number of rats	Number with muscular dystrophy	
21–2	2	2	3	0	600 mg. wheat germ oil
36–2	2	2	3	0	600 mg. wheat germ oil
80–2	3	3	2	0	600 mg. wheat germ oil
64	2	1	2	0	600 mg. wheat germ oil
80–2	3	3	2	1	300 mg. wheat germ oil
80–3	2	1	2	0	300 mg. wheat germ oil
29–2	3	3	3	0	300 mg. wheat germ oil
8–2	3	2	2	0	300 mg. wheat germ oil
35–2	2	2	2	0	300 mg. wheat germ oil
77	3	2	2	0	300 mg. wheat germ oil
76	3	3	3	0	300 mg. wheat germ oil
35–1	2	2	3	0	600 mg. of oil of treated wheat germ
9	2	2	3	0	600 mg. of oil of treated wheat germ
12	2	2	3	0	600 mg. of oil of treated wheat germ
36–2	2	2	3	0	600 mg. of oil of treated wheat germ
80–3	2	1	3	1	300 mg. of oil of treated wheat germ
45	3	3	2	2	300 mg. of oil of treated wheat germ
8–2	3	2	3	0	300 mg. of oil of treated wheat germ
35–2	2	2	2	1	300 mg. of oil of treated wheat germ
36–3	2	2	2	2	300 mg. of oil of treated wheat germ
37–2	2	2	1	0	5 mg. α-tocopherol
54	1	1	2	0	5 mg. α-tocopherol
77	3	2	3	0	5 mg. α-tocopherol
76	3	3	3	0	5 mg. α-tocopherol
65	3	1	3	0	5 mg. α-tocopherol
7–3	3	3	3	3	0.5 mg. α-tocopherol
66–2	3	3	3	3	0.5 mg. α-tocopherol
29–3	2	1	2	1	0.5 mg. α-tocopherol
67–2	3	3	3	1	0.5 mg. α-tocopherol

were subsequently born to most of them after administration of wheat germ oil during the gestation period.

Since 1 gm. of wheat germ oil permitted normal pregnancies in rats on an E-low diet and since the oil from the treated wheat germ failed, even when fed in amounts as great as 20 gm. per gestation, it is clear that the ferric chloride treatment had inactivated most of the vitamin E. These results are in agreement with those of Waddell and Steenbock ('31), who obtained a marked inactivation of vitamin E in wheat germ by treating the germ with an ether solution of ferric chloride. However, these authors found only a partial destruction of vitamin E activity in samples of wheat germ oil that had been treated with ethereal ferric chloride.

α-Tocopherol. The α-tocopherol was dissolved in 47 parts of ethyl laurate and 1 drop of the solution given daily for 15 days to each of twelve rats in five litters. A total amount of 5 mg. of α-tocopherol was given each rat. The controls in these litters were given daily 1 drop of ethyl laurate. All rats receiving the α-tocopherol were protected. The remainder of the solution of α-tocopherol was diluted with 9 parts of ethyl laurate and 1 drop of the resulting solution was fed daily for 15 days (total dose 0.5 mg.) to eleven rats in four litters. Only three rats were protected.

DISCUSSION

Since muscular dystrophy in rats occurs upon diets that are known to be low in vitamin E, and since protection is afforded by wheat germ oil and α-tocopherol, it would appear that the muscle lesions are due to a deficiency of the antisterility vitamin. However, the results with oil of treated wheat germ, which in adequate dosage prevented degeneration of muscle in young rats but failed to facilitate the birth of living young in adult females, raise certain new problems.

There are several possible explanations for this discrepancy:

1. The requirement of vitamin E for successful reproduction in an adult female may be much greater than that of the young rat for normal muscular development.

2. The young rat may differ in its ability to utilize possible precursors for vitamin E synthesis.

3. Several substances are known to have vitamin E activity (Evans, Emerson and Emerson, '38). There may be several substances that protect against muscular dystrophy, and it is conceivable that the α-tocopherol employed may have contained impurities that are active in preventing the muscle disease.

Inasmuch as the onset of the lesions of nutritional muscular dystrophy in Herbivora and ducklings is prevented or delayed by the inclusion of vegetable oils in the diet (Madsen, '36; Pappenheimer and Goettsch, '36; Mattill, '38), it will be of interest to feed α-tocopherol to these species.

The observation that muscle dystrophy occurs in young rats on synthetic diets low in vitamin E, but not on natural foods diets in which the vitamin E has been destroyed by ethereal ferric chloride, is confirmed by similar experiments upon goats. Thomas and Cannon ('37) fed a treated natural foods diet, on which male rats became sterile in 5 months and females presented typical resorption gestations, to a herd of goats for 4½ years. Normal third generation kids were produced. On the other hand when Madsen, McCay and Maynard ('35) fed kids from the time of weaning on a diet composed of regenerated cellulose, casein, sucrose, starch, yeast, salt mixture and lard, the animals died after 4½ to 10 months. Histological studies showed skeletal muscle lesions in all of the animals.

The contention of Morgulis, Wilder and Eppstein ('38) that two factors, vitamin E and B complex, are necessary for the prevention of muscular dystrophy in rabbits does not apply to the rat disease.

SUMMARY

A diet is described on which 90% of rats at the end of lactation suddenly become affected with severe degeneration of the skeletal muscles.

A method is described for testing the protective effect of preparations against muscular dystrophy.

Young rats are protected against the muscle disease by supplementing the diet with wheat germ oil, with oil of treated wheat germ in which the anti-sterility vitamin has been inactivated by ethereal ferric chloride or with α-tocopherol.

The activity of a preparation in preventing muscular dystrophy does not necessarily correspond to its anti-sterility potency.

LITERATURE CITED

BURR, G. O., W. R. BROWN AND R. L. MOSELEY 1937 Paralysis in old age in rats on a diet deficient in vitamin E. Proc. Soc. Exp. Biol. and Med., vol. 36, p. 780.

EINARSTEN, L., AND A. RINGSTED 1938 Effect of chronic vitamin E deficiency on the nervous system and the skeletal musculature in adult rats. A neurotropic factor in wheat germ oil. Nut. Abstracts, vol. 8, Abstract 286, p. 52.

EVANS, H. M., AND G. O. BURR 1927 The anti-sterility fat soluble vitamine E. Memoirs of the University of California, Berkeley, vol. 8.

——— 1928 Development of paralysis in the suckling young of mothers deprived of vitamin E. J. Biol. Chem., vol. 76, p. 273.

EVANS, H. M., O. H. EMERSON AND G. A. EMERSON 1936 The isolation from wheat germ oil of an alcohol, α-tocopherol, having the properties of vitamin E. J. Biol. Chem., vol. 113, p. 319.

EVANS, H. M., G. A. EMERSON AND O. H. EMERSON 1938 The chemistry of vitamin E. II. Biological assays of various synthetic compounds. Science, vol. 88, p. 38.

EVANS, H. M., G. A. EMERSON AND I. R. TELFORD 1938 Degeneration of cross-striated musculature in vitamin E low rats. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 625.

GOETTSCH, M., AND E. F. BROWN 1932 Muscle creatine in nutritional muscular dystrophy of the rabbit. J. Biol. Chem., vol. 97, p. 549.

GOETTSCH, M., AND A. M. PAPPENHEIMER 1931 Nutritional muscular dystrophy in the guinea pig and rabbit. J. Exp. Med., vol. 54, p. 145.

HAWK, P. B., AND B. L. OSER 1931 A modification of the Osborne-Mendel salt mixture. Science, vol. 74, p. 369.

KNOWLTON, G. C., AND H. M. HINES 1938 Effect of vitamin E-low diet upon skeletal muscle. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 665.

MADSEN, L. L. 1936 The comparative effects of cod liver oil, cod liver oil concentrates, lard and cottonseed oil in a synthetic diet on the development of nutritional muscular dystrophy. J. Nutrition, vol. 11, p. 471.

MADSEN, L. L., C. M. MCCAY AND L. A. MAYNARD 1935 Synthetic diets for Herbivora with special reference to the toxicity of cod liver oil. Cornell Univ. Agr. Exp. Sta., Memoir 178, p. 26.

MASON, K. 1933 Differences in testis injury and repair after vitamin A deficiency, vitamin E deficiency and inanition. Am. J. Anat., vol. 52, p. 153.

MATTILL, H. A. 1938 Vitamin E and nutritional muscular dystrophy in rabbits. Abstracts XVI Internat. Physiol. Kongress, p. 112.

MORELLE, J. 1931 Influence de la privation de la vitamin E chez le rat au course de la lactation. C. R. Soc. Biol. Paris, vol. 108, p. 804.

MORGULIS, S., AND H. C. SPENCER 1936 A study of the dietary factors concerned in nutritional muscular dystrophy. J. Nutrition, vol. 11, p. 573.

MORGULIS, S., V. M. WILDER AND S. H. EPPSTEIN 1938 Further studies on dietary factors associated with nutritional muscular dystrophy. J. Nutrition, vol. 16, p. 219.

OLCOTT, H. S. 1938 The paralysis in the young of vitamin E deficient female rats. J. Nutrition, vol. 15, p. 221.

OLCOTT, H. S., AND H. A. MATTILL 1934 Vitamin E. I. Some chemical and physiological properties. J. Biol. Chem., vol. 104, p. 423.

PAPPENHEIMER, A. M. 1939 The pathology of nutritional muscular dystrophy of young rats. Am. J. Path., in press.

PAPPENHEIMER, A. M., AND M. GOETTSCH 1936 Transmission of nutritional muscular dystrophy to rabbits in utero. Proc. Soc. Exp. Biol. and Med., vol. 34, p. 522.

RINGSTED, A. 1935 A preliminary note on the appearance of paresis in adult rats suffering from avitaminosis E. Biochem. J., vol. 29, p. 788.

ROSE, W. C., O. M. HELMER AND A. CHANUTIN 1927 A modified method for the estimation of total creatinine in small amounts of tissue. J. Biol. Chem., vol. 75, p. 543.

THOMAS, B. H., AND C. Y. CANNON 1937 Reproduction on rations free from vitamin E. Proc. Am. Soc. Animal Prod., p. 59.

WADDELL, J., AND H. STEENBOCK 1928 The destruction of vitamin E in a ration composed of natural and varied foodstuffs. J. Biol. Chem., vol. 80, p. 431.

———— 1931 Vitamin E in iron treated dry rations. J. Nutrition, vol. 4, p. 79.

ELECTROLYTE AND NITROGEN METABOLISM IN PREGNANCY

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FOUR FIGURES

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The very fact that nature provides a mechanism for temperature control with loss of water and salts through the skin makes electrolyte balance measurements only approximations even when the subjects carry on a sedentary existence. When the subjects are engaged in the activities of daily life, under varying conditions of temperature and humidity, how much more variable must be the losses of these substances! Because of the vagaries of human nature the most careful and continual surveilance is almost an absolute requisite in order to preclude all but the unusual subject from taking liberties that she might regard as inconsequential in effect. The quantitative determination of sodium and potassium is not one of the more simple chemical procedures. And yet, precise as are the analytical methods used in some of the experiments reported in the literature, it is felt that some of the studies have lost much of their potential value because the subjects assumed a large share of the responsibility in the preparation of the diet and collection of excreta.

Coons and co-workers ('34) observed an average daily retention of 1.26 gm. of sodium and 0.51 gm. of potassium in pregnant women who lived in their domestic environments and subsisted on a more or less self chosen diet. Hummel and co-workers ('36) observed an average daily retention of 0.56 gm. of sodium and 1.40 gm. of potassium over a prolonged period during pregnancy. The subject's diet, however, was self chosen. The data of Freyberg, Reekie and Folsom ('38) show an average daily retention of 0.17 gm. of sodium, allowance being made for loss through the skin. Coons and Hummel reported average daily retentions of 0.89 gm. and 0.60 gm. of chloride respectively. Evidence cited by Wilson ('16) and Stander ('36) indicates that nitrogen is stored in pregnancy, often in excess of the needs of the fetus. Nitrogen balance studies were included in the present investigation to determine any relationship between the storage of this element and that of potassium, sodium and chloride.

GENERAL METHODS

Care of patient. These studies were conducted on two women. Observations on one of them were made during the fourth and eighth months of pregnancy over 10- and 11-day periods respectively. The other subject had mild symptoms of toxemia with moderate edema and elevation of blood pressure. On her the observation period had to be terminated after 5 days because of the onset of labor at term. These subjects were maintained in a special study division under practically constant supervision and observation by nurses trained in metabolic work. On admission a complete physical and laboratory examination was made. Three days were allowed for the subjects to accustom themselves to the diet and other matters of strict routine before actual determinations were begun. The usual bedroom diversions were permitted, but exercise more vigorous than walking slowly was not allowed. At no time was visible perspiration noted even in the axillae.

The study periods began and ended at 8 o'clock A.M. on consecutive days. At 8 o'clock A.M. after voiding, the subjects were weighed on a balance sensitive to 10 gm. Blood samples were then taken as needed and weighed. A given amount of weighed food and distilled water was given at regular intervals, i.e., 8.30 A.M., 1.30 P.M. and 6 P.M., care being taken that the entire diet including the rinsings of the dishes with distilled water from the total alotted quota, and nothing else, was consumed.

Clinical histories. Experiment I. H.R., no. 115937. The subject was a 28-year-old primigravida in her fourth month of pregnancy. Her past medical history indicated good average health. Her pregnancy likewise had progressed entirely normally. Examination showed her to be in excellent physical condition. The blood counts and urinary findings were likewise normal. The B.M.R. however was —17%. Metabolic period began on June 22, 1936. Weight 53.21 kg. Diet was well tolerated. Stools were passed every second day and were sharply demarcated with carmine. Study completed on July 2, 1936. Weight 53.19 kg.

Experiment II. Same subject as in experiment I, but now in the eighth month of pregnancy. Interval note: The pregnancy had progressed without toxic manifestations or other complications. Examination showed her to be in good health. Metabolic period began on September 25, 1936. Weight 57.16 kg. B.M.R., —18%. Diet well tolerated. Study period of 11 days terminated on October 6, 1936. Weight 57.21.

Experiment III. D.V., no. 12516. This subject, a 40-year-old para 1, was admitted 7 days prior to her expected date of confinement. Her past health had been good and there was no history of serious illness. In early pregnancy she had moderately severe nausea and vomiting. Several days before admission her blood pressure rose from 100/60 to 152/88, and for the first time ankle edema was noticed. A mild albuminuria was present. The patient appeared to be a suitable subject typifying pre-eclampsia. Examination showed her to be in good general physical condition. Blood pressure 158/85. Moderate pre-tibial edema present. Blood counts normal. Blood N.P.N. 26 mg.%. The urine contained about ¼ gm. of albumin per 24-hour specimen, but was otherwise normal. B.M.R. +12%. Metabolic period began October 9, 1936. Weight 73.54. Diet well taken, but subject had numerous diarrhoeal stools. Complete data over a 5-day period were available, but the study had to be subsequently terminated because of the onset of labor. Weight at close of period 72.97 kg.

Diet. The standard daily diet in each of the three experiments was as follows:

Distilled water	2500 gm.
Powdered whole milk	252 gm.
Lactose	36 gm.
Chocolate malted milk	20 gm.
Graham crackers	50 gm.
Tomato juice	400 gm.
Dextrin	20 gm.
Sucrose	10 gm.
Sodium chloride, 10% sol.	30 cc.

These foods were of standard brands and were pooled from containers having the same lot numbers. They were prepared in sufficient quantities to insure against the need of replenishment from outside sources during the course of the experiment and stored in the refrigerator. The absolute uniformity obviated the need of repeated analyses. The composition of the stools was consequently likewise quite uniform. This diet contained protein approximately 70 gm., fat 77 gm., carbohydrates 230 gm., calcium 2.17 gm., phosphorus 1.84 gm., iron 0.005 gm., and was complete as regards vitamins A, B, C and G. All items of the diet, in each experiment were analysed for moisture, potassium, sodium, chlorides and nitrogen.

Collection of blood. Blood was withdrawn by vena-puncture without stasis and collected under oil after a 12-hour fast at the beginning and conclusion of a metabolic period. The serum was analysed for Na, K, Cl, N and total base.

Collection of excreta. Urine. The urine was passed directly into a bottle packed in ice and containing 10 cc. of glacial acetic acid. Aliquots from the mixed 24-hour specimen were analysed for Na, K, Cl and N. Stools. The stools were sharply demarcated with carmine and stored in a vessel containing 10 cc. of glacial acetic acid and a measured amount of distilled water. All stools not relating to the experiment were discarded. Because of the irregularity of movement of the bowls, the total fecal constituents passed over the entire metabolic period were pro-rated on a daily basis. The stool-water mixture was subjected to prolonged mechanical agitation and analysed for Na, K, Cl and N.

Analytical methods. Sodium was determined by the method of Barber and Kolthoff ('28) as modified by Salit ('32), and potassium by the method of Shohl and Bennett ('28) as modified by Hald ('33). Chloride in the food was determined by the method of Birner ('28); and in the urine and feces by a modified Volhard-Harvey titration. Total base was determined by the method of Keys ('36). Nitrogen was determined by the Kjeldahl procedure.

RESULTS AND DISCUSSION

The data are presented in table 1. An advantage of the diet used is indicated by the constant daily input of electrolyte and nitrogen. Variations in the output of these elements must therefore have another basis. With this diet, the subjects maintained their approximate weights. A small amount of weight loss frequently occurs just prior to the onset of labor at term. The weight loss in experiment III may have been caused by excessive water loss due to diarrhoea.

The data indicate a discernable retention of sodium. The average daily retentions of sodium were found to be 19.3 m. eq. in the fourth month and 11.6 m. eq. and 13.2 m. eq. in the eighth and ninth months, respectively. The average daily retention values for potassium were 8.4, 10.4 and 5 m. eq. in the same periods. No constant upward or downward trend is noted in the curve for chloride accumulation, the average daily balances of chloride being —5.5, +1.2 and +4 m. eq., respectively. Apparently some other anion than chloride must be linked with the stored potassium.

It is noted that the sum of the sodium and potassium in the serum approximates the values for total base. But when one considers from the calibration of the methods that sodium recovery was 2.7% high and total base recovery was 1.3% low, the apparent discrepancy between sodium, potassium and total base is largely removed. Appreciable changes in serum electrolytes were not observed.

TABLE 1
Daily electrolyte and nitrogen balances

EXPERIMENT	DATE	WT.	REL. HUM.	TEMPERATURE °F.	INPUT				OUTPUT				BALANCE				SERUM PER LITER				
					Na	K	Cl	N	Na	K	Cl	N	Na	K	Cl	N	Na	K	Cl	Total base	N
		kg.	%		*m eq.*	*m. eq.*		*gm.*	*m. eq.*	*m. eq.*		*gm.*	*m. eq.*	*m. eq.*		*gm.*	*m. eq.*	*m. eq.*			*gm.%*
No. 1 H.R. 4 months pregnancy	6/22/36	53.21	48	72	158	105	164	11.23	141	98	201	12.48	17	7	—37	—1.25	141.0	4.7	104.6	145.1	1.07
	6/23/36	53.27	51	72	158	105	164	11.23	141	98	178	12.20	17	7	—14	—0.97					
	6/24/36	53.03	55	71	158	105	164	11.23	141	98	168	12.35	17	7	—4	—1.12					
	6/25/36	53.29	57	73	158	105	164	11.23	139	97	129	11.83	19	8	35	—0.60					
	6/26/36	53.34	67	76	158	105	164	11.23	117	96	147	12.29	41	9	17	—1.06					
	6/27/36	53.45	72	77	158	105	164	11.23	124	97	160	11.02	34	8	4	0.21					
	6/28/36	53.38	56	74	158	105	164	11.23	157	101	182	11.36	1	4	—18	—0.13					
	6/29/36	53.16	57	72	158	105	164	11.23	150	89	186	10.94	8	16	—22	0.29					
	6/30/36	53.38	53	73	158	105	164	11.23	127	85	156	11.31	31	20	8	—0.08					
	7/1/36	53.19	53	72	158	105	164	11.23	150	107	188	11.48	8	—2	—24	—0.25					
	7/2/36																142.4	..	102.1	144.7	1.03
										Total retention			193	84	—55	—4.96					
										Av. daily ret.			19.3	8.4	—5.5	—0.50					
No. 2 H.R. 8 months pregnancy	9/25/36	57.16	51	75	145	107	166	11.42	134	92	174	11.58	11	15	—8	—0.16	139.8	5.5	106.3	145.0	0.96
	9/26/36	57.20	51	77	145	107	166	11.42	135	104	177	11.40	10	3	—11	0.02					
	9/27/36	57.41	65	77	145	107	166	11.42	119	103	156	10.96	26	4	10	0.46					
	9/28/36	57.33	53	77	145	107	166	11.42	120	95	163	11.02	25	12	3	0.40					
	9/29/36	57.28	48	78	145	107	166	11.42	137	98	170	10.79	8	9	—4	0.63					
	9/30/36	57.34	49	78	145	107	166	11.42	116	95	147	11.69	29	12	19	—0.27					
	10/1/36	57.04	56	79	145	107	166	11.42	165	101	181	12.08	—20	6	—15	—0.66					
	10/2/36	57.06	53	79	145	107	166	11.42	144	89	166	11.31	1	18	0	0.11					
	10/3/36	57.16	51	78	145	107	166	11.42	156	93	175	11.38	—11	14	—9	0.04					
	10/4/36	57.09	46	78	145	107	166	11.42	122	93	150	10.55	23	14	16	0.87					
	10/5/36	57.21	55	80	145	107	166	11.42	119	100	154	10.28	26	7	12	1.14					
	10/6/36																141.2	5.4	106.3	145.3	1.04
										Total retention			128	114	13	2.56					
										Av. daily ret.			11.6	10.4	1.2	0.23					
No. 3 D.V. term pregnancy	10/9/36	73.54	60	75	146	102	168	11.53	126	92	160	14.21	20	10	8	—2.68	142.4	5.4	107.0	146.6	0.96
	10/10/36	73.39	66	74	146	102	168	11.53	122	96	156	15.08	24	6	12	—3.55					
	10/11/36	73.21	56	73	146	102	168	11.53	123	100	147	13.40	23	2	21	—1.87					
	10/12/36	73.40	47	74	146	102	168	11.53	146	103	180	12.35	0	—1	—12	—0.82					
	10/13/36	72.97	50	74	146	102	168	11.53	147	94	177	12.82	—1	8	—9	—1.29					
	10/14/36																140.8	5.6	105.6	145.8	0.93
										Total retention			66	25	20	—10.21					
										Av. daily ret.			13.2	5	4	—2.04					

The positive balance of electrolytes illustrated in figure 3 is contrary to what might be expected of a diarrhoeal patient losing ½ kg. in weight. The subject in this experiment was edematous, but it is difficult accurately to measure weight loss due to reduction of edema. Can it be that the demands for electrolytes during pregnancy are strong enought to withstand the consequence of a mild diarrhoea? Although our data do not support the generally accepted evidence of nitrogen storage, it is not to be regarded as a refutation of that evidence. Results must be interpreted as they relate to the conditions of an experiment. Sherman ('26) has emphasized that the body tends to adjust its protein metabolism to its protein supply, and that an appreciable length of time may be required to effect adjustment from a higher to a lower level. Perhaps in these experiments, the relative nitrogen deficit resulted from adjustments to lower protein intake than that to which the subjects had been accustomed. The passage of unassimilated nitrogen in the stool, in experiment III, may have contributed in large measure to the nitrogen deficit. In any event, the subject lost an average of 2.83 gm. of nitrogen daily in the stools, while the subject in experiments I and II lost only 0.95 gm. of N daily by this route.

Daily variations in the balances of electrolytes were noted in all three experiments and are illustrated in figure 4. The sodium and chloride balances in experiment II are seen to parallel one another quite closely; in fact, for 5 days the retentions of these elements were equivalent. This striking parallelism was likewise noted in experiments I and III. Potassium balances varied less than either those of sodium or chloride. There appeared to be no correlation between the potassium and the sodium variation. The variations were probably referable to such unmeasured factors as changes in exercise, humidity and temperature. Freyburg and Grant ('37), using as subjects "men who went about their usual laboratory duties with special precautions only to keep themselves cool enough to prevent sweating" found the daily loss through the skin to be sodium 71 to 209 mg., potassium 133

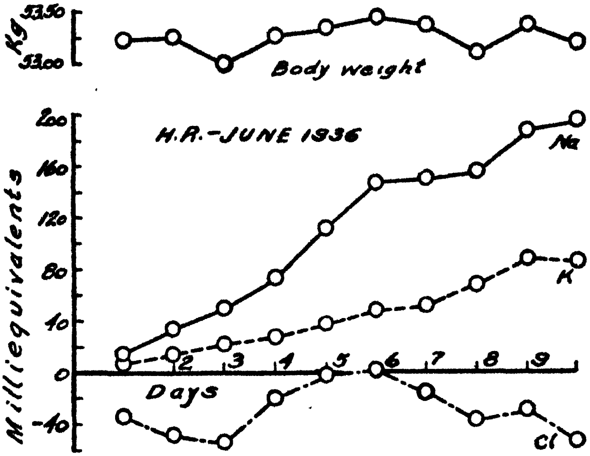

Fig. 1 Showing the cumulative balances in experiment I. Subject H.R., 4 months pregnant.

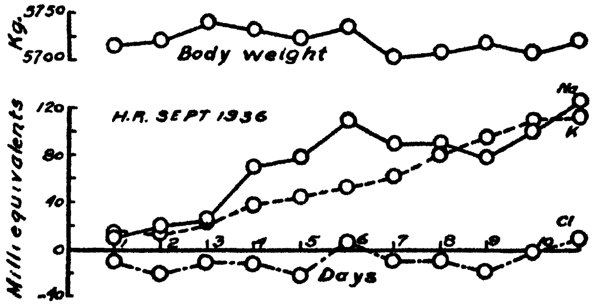

Fig. 2 Showing the cumulative balances in experiment II. Subject H.R., 8 months pregnant.

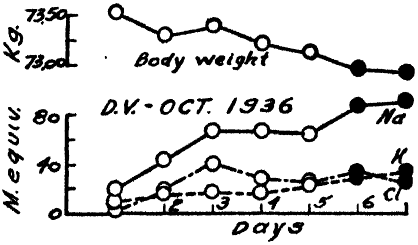

Fig. 3 Showing the cumulative balances in experiment III. Subject D.V., at term. The data for the last 2 days are incomplete because of the sudden onset of labor on the seventh day.

to 179 mg. and chloride 146 to 248 mg. Basset and Keutmann (unpublished data) studied arthritic subjects and found daily losses through the skin to be sodium 180 mg., potassium 239 mg. and chloride 194 mg. daily.

Considerable variation in output of these electrolytes through the skin is thus noted, and while it would be jeopardous to transpose data from one type of experiment to another, it shows the approximate magnitude of electrolyte loss with which we might be expected to contend. It is felt that even when allowance is made for losses through the skin, the evidence presented in this paper suggests definite storage of sodium. The evidence for potassium and chloride storage is less conclusive.

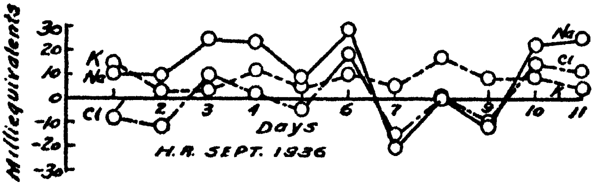

Fig. 4 Showing diurnal variations in retention of electrolytes in experiment 2. Experiments I and III likewise show comparable variations.

SUMMARY

Electrolyte and nitrogen balance studies were conducted under practically constant supervision on two women in the fourth, eighth and ninth months of gestation. The same diet was used in each experiment and was of constant composition from day to day. Diregarding losses through the skin, the average daily retentions of sodium and potassium based on the three metabolic studies were observed to be 14.7 milliequivalents and 7.9 milliequivalents respectively. Fluctuations in potassium retention varied less from day to day than did those of sodium and chloride. Variations in sodium and chloride retention appear to parallel one another. Such a parallelism does not seem to apply to potassium. The subjects were maintained in approximate chloride equilibrium. For only one experiment was a positive nitrogen balance observed. The nitrogen deficit in one of the experiments may be explained on the basis of diarrhea. The need of prolonged periods of continuous observation is stressed.

The authors are happy to acknowledge with gratitude the assistance given in this study by Miss Clara Miller, R.N. and Mrs. Verna Eisenhauer, R.N. Dr. Estelle Hawley has our appreciation for the planning of the diet.

LITERATURE CITED

BARBER, H. H., AND I. M. KOLTHOFF 1928 A specific reagent for the rapid gravimetric determination of sodium. J. Am. Chem. Soc., vol. 50, p. 1625.

BASSETT, S. H., E. H. KEUTMANN, S. L. WARREN AND H. E. VAN ALSTYNE. (Unpublished data.)

BIRNER, M. 1928 Eine verbesserte Methode zur Chlorbestimung in Organen und Nahrungsmitteln. Zeit. f. ges. Med., Bd. 61, S. 700.

COONS, C. M., R. R. COONS AND A. T. SCHIEFELBUSCH 1934 The acid base balance of the minerals stored during human pregnancy. J. Biol. Chem., vol. 104, p. 757.

FREYBERG, R. H., AND R. L. GRANT 1937 Loss of minerals through the skin of normal humans when sweating is avoided. J. Clin. Invest., vol. 16, p. 729.

FREYBERG, R. H., R. D. REEKIE AND C. FOLSOME 1938 A study of the water, sodium and energy exchange during the latter part of pregnancy. Am. J. Obs. and Gyn., vol. 36, p. 200.

HALD, P. M. 1933 The determination of the bases of serum and whole blood. J. Biol. Chem., vol. 103, p. 471.

HUMMEL, F. C., H. R. STERNBERGER, H. A. HUNSCHER AND I. G. MACY 1936 Metabolism of women during the reproductive cycle. VII. Utilization of inorganic elements. J. Nutrition, vol. 11, p. 235.

KEYS, A. 1936 The determination of total base in blood and other biological fluids by the electrodialysis method of Adair and Keys. J. Biol. Chem., vol. 114, p. 449.

SALIT, P. W. 1932 A new triple acetate method for sodium determinations in biological materials. J. Biol. Chem., vol. 96, p. 659.

SHERMAN, H. C. 1926 Chemistry of Food and Nutrition. Third ed. The MacMillan Co., New York, p. 236.

SHOHL, A. T., AND H. B. BENNETT 1928 A micro method for the determination of potassium as iodoplatinate. J. Biol. Chem., vol. 78, p. 643.

STANDER, H. S. 1936 Williams Obstetrics. D. Appleton-Century Co., New York.

WILSON, K. M. 1916 Nitrogen metabolism during pregnancy. Johns Hopkins Hosp. Bull., vol. 27, no. 303, p. 121.

A QUANTITATIVE STUDY BY MEANS OF SPECTROGRAPHIC ANALYSIS OF ALUMINUM IN NUTRITION [1]

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ONE FIGURE

(Received for publication November 30, 1938)

Whether aluminum is present in plant and animal matter formed the basis of a controversy between McCollum, Rask and Becker ('28) and Kahlenberg and Closs ('29). Both used the spectrographic technic in their studies. The former used only the spark to obtain the emission of light from the elements, while the latter used both the arc and spark methods of exciting the elements. McCollum, Rask and Becker ('28) concluded from the inspection of their spectrograms that aluminum is a constituent neither of plant nor animal matter. Kahlenberg and Closs ('29) on the other hand reported the presence of aluminum in these materials when both the spark and arc were employed. Whether the length of the arcing or sparking period was an influencing factor in determining the presence of aluminum in these materials is not known from the published data. Recent work of Harrison ('36) indicates that the different elements require different lengths of excitation time before emitting light. He found that the aluminum lines were much more intensified after 80 seconds of excitation. The findings of Kahlenberg and Closs, however, have been confirmed by Underhill and Peterman ('29) and by Cox and co-workers ('32). The earlier literature which has been summarized by Smith ('28) indicates that aluminum is widespread in nature.

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[1] The data of this study are taken from a dissertation submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in nutrition, Child Welfare Research Station, State University of Iowa.

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According to Lewis ('31) human blood is free from aluminum or contains only 1 part in five or ten millions, while Underhill and Peterman ('29) reported that aluminum is always present in blood of normal man, the average concentration being 0.060 mg. per 100 ml. of blood. Most investigators agree that there is no convincing evidence that small amounts of aluminum are harmful to the normal individual, but that large amounts may be. However, Myers and Mull ('28) found that when 8 mg. of aluminum per kilogram of body weight, two to three times the amount the Referee Board ('14) considered a large amount, was given rats the minute traces of aluminum normally present in tissues was only slightly increased. Furthermore, Tocco and Mulas ('35) concluded from their work that aluminum powders produced by mechanical cleaning of cooking utensils did not have injurious effects when ingested or injected into frogs and rabbits.

All evidence to date is to the effect that aluminum in the amounts present in foods, either normally or after cooking is harmless. There is little evidence to show whether or not it is an essential constituent of the daily diet. It seemed possible that this might be determined through balance studies with children.

The purpose of the present study was to determine the amount, if any, of aluminum retained by children of preschool age. The spectrograms of the ashed food, feces, urine, distilled water and acid alcohol were compared with spectra produced by solutions of known aluminum content.

MATERIALS AND METHODS

The diets employed, the care used in handling and collecting of the food and excreta, the preparation of aliquots for spectrographic analysis and the method used in reading the spectrograms is given by Scoular ('38). A portion of that description is repeated here.

During the entire study only two major changes were made in the original diet (diet I) which consisted of a whole grain bread and cereal, butter, green beans, carrots, potatoes, bananas, apple sauce, prunes, eggs, ground beef and milk. Each child also received specified amounts of Viosterol, cod liver oil and orange juice daily. The first change (diet II) consisted in substituting white bread and Cream of Wheat for the whole grain bread and cereal, tomatoes for green beans, and decreasing the amount of orange juice by one-half. The second change (diet III) consisted in a reduction in the amount of meat and egg and an increase in the quantity of fruit and vegetables and butter. Whenever possible, the necessary adjustments for the increased caloric needs of the child were made by slight changes in sugar.

The duplicate 4 gm. samples of dried food first used were increased to 6 gm. when it was found that the smaller samples gave spectrographic lines for copper which were less distinct and more difficult to read. Two grams of the dried feces and 500 ml. of urine were used throughout the study. Both the food and the feces were ashed for 12 hours at dull red heat in a muffle furnace, whereas the urine was ashed in the air with the aid of two Fischer burners. Five hundred milliliter samples from each carboy used of distilled water, as well as acid-alcohol were evaporated and ignited prior to dilution.

Each of the ashed samples was dissolved in 3 ml. of hydrochloric acid and made up to a volume of 10 ml. in calibrated Pyrex volumetric flasks with redistilled water.

A known weight of aluminum which had been precipitated by 8-hydroxy-quinoline[3] was dissolved in hydrochloric acid and made up to a given volume with redistilled water. The difference between the length of the aluminum line, 3961.5 Angstroms, and the reference bands, 3889 Angstroms, was read from spectrograms which were made from graded concentrations of this solution. These differences were plotted for a given concentration and used in determining the amount of aluminum in the unknowns.

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[3] Method taken from Organic Reagents for Metals, Staff Research Laboratory of Hopkins and Williams, Ltd., 1933.

The effect of the sodium chloride, present in the unknown solutions, upon the length of both the copper and zinc lines (Scoular, '38, '39) suggested that it might have an effect upon the aluminum line. The influence of sodium chloride, therefore, was tested. Two sets of electrodes were prepared from the special spectrographic quality graphite, to each of which was added the same amount of aluminum. To one of these there were added in addition to the aluminum 4 drops of a saturated solution of sodium chloride. When the spectrograms of these two sets of electrodes were compared, it was found that the sodium chloride produced less difference between the aluminum line 3961.5 Angstroms and the reference bands at 3889 Angstroms, than was obtained from the spectra of aluminum alone. This effect was similar to that experienced with copper and zinc when sodium chloride was arced with these. Therefore, both aluminum lines were plotted for a given concentration (fig. 1). When 4 drops of sodium chloride were used it was possible to read a concentration of 0.00005 mg. of aluminum per milliliter, while without the addition of the sodium chloride to the electrode, 0.0005 mg. was the lowest concentration at which this wave length could be read. The effect of the sodium chloride therefore, was to lessen the distance between the aluminum line 3961.5 Angstroms and the reference bands at 3889 Angstroms.

Since the wedge spectrograms used in the copper study were also used to obtain the aluminum retentions, all precautions which were employed to prevent contamination with copper, also prevented the addition of extraneous aluminum.

The most sensitive aluminum line 3961.5 Angstroms was used to read both the standards and the unknowns since it was the only aluminum line appearing on all the wedge spectrograms. Because of the previously mentioned effect of sodium chloride upon the difference between the aluminum line and the reference bands, the concentration of the aluminum in the

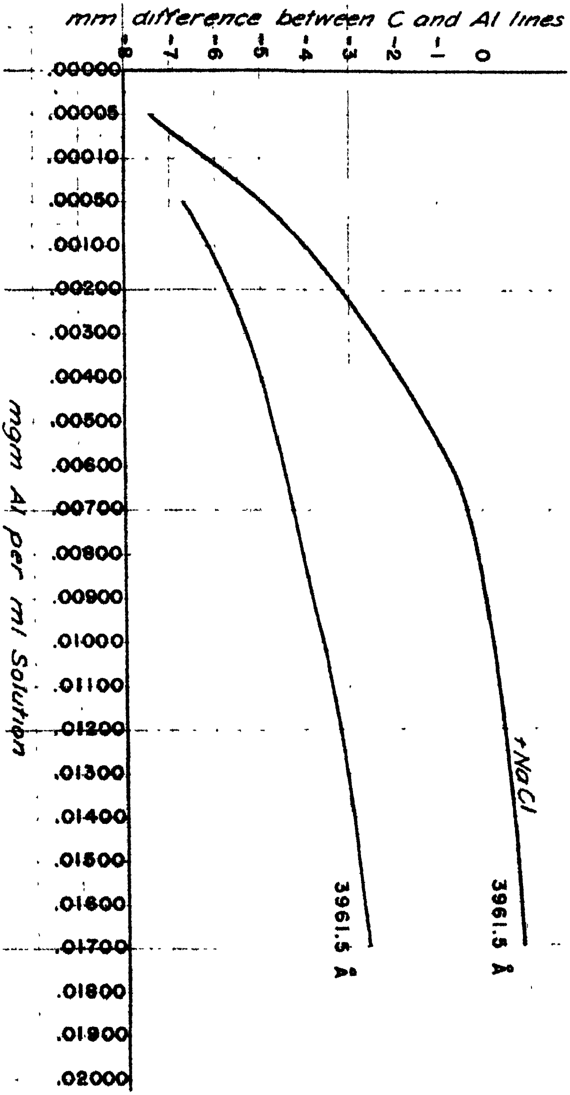

Fig. 1 Aluminum concentration, in milligrams plotted for each tenth of a millimeter difference between the length of the zinc line and the reference bands, in spectra produced by standard solutions, with and without the addition of sodium chloride.

unknowns was determined as for copper (Scoular, '38) by reading the 'best-fit' curves.

The spectrograms of the distilled water used for drinking purposes showed the water to contain a variable amount of aluminum, namely 0.001 to 0.015 mg. per 500 milliliters. The cause of the wide range of aluminum content of the distilled water may be explained by the length of time the water stood in the carboys, or by the fact that from time to time new carboys may have been used. The acid-alcohol used on the feces and food, however, contributed from 0.001 to 0.003 mg. per 100 ml. Since in all cases the water used was tested and measured, these findings did not invalidate the results.

The 'intensity' of the food spectra was very indistinct; this combined with the incandescent effect of the graphite electrode on the spectrogram made it difficult to read the end of the line. Lamb ('35) also observed this effect. Three or more readings of each line were necessary. The average of these was used in comparing the differences between the aluminum line and the carbon bands of the duplicate spectrograms made of each aliquot.

To test the method employed in this study, 0.005 mg. of aluminum was added to duplicate electrodes of food, feces and urine. Recoveries of from 100 to 110%, 90 to 106% and 90 to 96%, respectively, were obtained (table 1). These findings are much closer than those of Dee and Rask ('31) who reported ±25% error for the spark method. This wide range with the spark method, Lampitt and Sylvester ('32) attribute to the variability of the spark. In the above report of Dee and Rask, the authors suggest tests with the arc as being especially adapted to the analysis of biological materials. However, the ±10% error of recovery reported for the present study represents the recovery of 0.005 mg. of aluminum from samples containing 0.00065 to 0.0057 mg. of aluminum. Recoveries reported by Eveleth and Myers ('36) for the colorimetric methods, aurin and alizarin, were 80 to 95% for alizarin and 80 to 120% for aurin. These represented in all but one instance, the recovery of larger amounts of aluminum by the aurin method from samples containing from 0.0046 to 0.013 mg. of aluminum. This amount represents from 0 to 4 times as much as was used in the present study. Although the recoveries obtained with the alizarin method were made with the addition of much smaller amounts of aluminum, 0.0005 to 0.002 mg., the analysis of dog tissue by these two colorimetric methods showed that the alizarin-lake gave much higher results than did the aurin-lake. However, Eveleth and Myers ('36) conclude that if all of the interfering substances are removed, comparable results are obtained with these two methods. The recovery results obtained with the spectrographic technic reported in this study indicates that it is more sensitive than the colorimetric methods commonly used in analyzing biological materials for aluminum.

TABLE 1

Amount of added aluminum recovered from samples of food, feces and urine

PERIODS	SAMPLES TESTED CONCENTRATION	SAMPLE PLUS 0.005 MG. Al		RECOVERY
		Concentration		
	mg.	*mg.*	*mg.*	%
Urine				
J.E. 2/9	0.0015	0.0060	0.0045	90
G.E. 3/22	0.0039	0.0085	0.0046	92
F.V. 3/17	0.0032	0.0080	0.0048	96
Feces				
J.E. 3/17	0.0057	0.0110	0.0053	106
G.E. 3/18	0.0012	0.0057	0.0045	90
F.V. 3/22	0.0021	0.0068	0.0047	94
Food				
J.E. 6/2	0.0007	0.0057	0.0050	100
G.E. 4/21	0.0010	0.0065	0.0055	110
F.V. 4/21	0.0010	0.0060	0.0050	100

RESULTS AND DISCUSSION

No attempt was made to vary the ingestion level of aluminum. This was found, however, to be from 0.014 to 0.030 mg. of aluminum per kilogram of body weight. The variation in the aluminum content of the diets varied less with the diet than with the difference in food supply. The effect of the use of different cooking vessels upon the aluminum content of certain foods was shown by Beal and co-workers ('32). In all instances foods cooked in aluminum utensils had a higher aluminum content than when the same food was cooked in glass. Since the same utensils, bright aluminum, were used for the preparation of all foods used in this study, the amount of aluminum varied only with the food source and its reaction with the metal. This is well illustrated by diet III (table 2) for periods 6/2 and 6/7 when the prepared food furnished from 0.016 to 0.019 mg. of aluminum per kilogram of body weight, while the same diet during periods 4/21 and 4/26 and 5/12 and 5/17 furnished from 0.023 to 0.030 mg. per kilogram. Since the only known difference in these food supplies was the use of 'new' potatoes during the 6/2 and 6/7 periods, it suggests that these contained less aluminum than the old potatoes previously used. Some difference is also shown in the aluminum content of diet II during two consecutive double metabolism periods, but the difference is less marked than with diet III, falling within the range of experimental error for the method employed. The theoretical values of all diets could not be obtained since the published tables of Underhill and Peterman ('29), Winter and Bird ('29), Beal and co-workers ('32) and Cox et al. ('32) were incomplete. Using the available data, diet III for F.V. 4/21 and 4/26 was estimated to contain 12.5 mg. of aluminum, 11.3 mg. of which was estimated as supplied by milk alone. These estimated values were very much higher than the values obtained by spectrographic analysis of the foods used in the diet.

In all instances the urine contained measurable amounts of aluminum, from 0.012 to 0.160 mg. per day. This did not increase with increased ingestion levels. There was a tendency, however, for the urine to show slight changes in aluminum concentration as the aluminum content of the diet changes from one 5-day period to the next successive 5-day period. A range of from 3 to 29% of the ingested aluminum in the present study was excreted in the urine. Flynn and

Inouye ('28) report that 30% of the ingested aluminum was excreted in the urine. These results suggest that aluminum is absorbed in part at least, and excreted by the kidneys.

The ingested aluminum is largely excreted through the alimentary tract, from 38 to 127% in the case of the children studied. Flynn and Inouye ('28) reported an average of 70% excreted in the feces, whereas MacKenzie ('30 and '31) recovered from 74.9 to 114% from the feces of rats and concluded that aluminum was quantitatively excreted through the alimentary tract of rats. During certain periods of the present study more aluminum was excreted than ingested. The source of this excessive fecal aluminum is not known since every precaution was taken to prevent contamination. All but one of the seven balances exhibiting aluminum elimination in excess of that ingested, occurred during the first 5-day metabolism period. There are, however, three instances when the fecal aluminum of the second 5-day period greatly exceeded that of the first period, yet fell well within the amount ingested. These particular cases seem to point to delayed aluminum elimination.

Fifty per cent of the balances are negative, ranging from 0.0003 to 0.0070 mg. per kilogram, with the positive retentions varying from 0.0003 to 0.0053 mg. per kilogram. Sixty per cent of all of these fell within the ±10% error as determined for the method used in the study. The remaining 40% of the aluminum balances do not suggest that aluminum is essential in the diets of preschool age children, since only three of the eight cases are positive. And, since there is no relation between the ingestion level and these slight positive balances, it would seem that aluminum is not essential in the human dietary.

SUMMARY

Thirty-five aluminum balance studies have been made with three normal boys of preschool age.

The ashed food, feces, urine, distilled water and acid-alcohol were analyzed spectrographically for aluminum by comparing

TABLE 2

Average daily intake, excretion and retention of aluminum

DIET NUMBER	CASE DATE	ALUMINUM INGESTION					ALUMINUM EXCRETION			RETENTION	
		Food	H_2O	A. A.	Total		Feces	Urine	Total	Total	Per kilogram
					mg.	Per kilogram					
		mg.	*mg.*	*mg.*			*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
II	F.V.										
	3/19	0.27	0.01	0.00	0.28		0.37	0.08	0.45	—0.17	
	3/22	0.29	0.01	0.00	0.30		0.17	0.09	0.26	0.04	
	Average				0.29	0.014				—0.07	—0.004
II	J.E.										
	3/17	0.27	0.01	0.00	0.28		0.48	0.09	0.57	—0.29	
	3/22	0.30	0.01	0.00	0.31		0.18	0.10	0.28	0.03	
	Average				0.29	0.015				—0.13	—0.007
II	G.E.										
	3/18	0.24	0.01	0.00	0.25		0.12	0.09	0.21	0.04	
	3/22	0.26	0.01	0.00	0.27		0.10	0.09	0.19	0.08	
	Average				0.26	0.015				0.06	0.003
III	G.E.										
	6/2	0.30	0.01	0.01	0.30		0.39	0.10	0.49	—0.19	
	6/7	0.24	0.01	0.01	0.24		0.11	0.06	0.17	0.07	
	Average				0.27	0.016				—0.06	—0.003
III	F.V.										
	6/2	0.39	0.01	0.01	0.39		0.25	0.12	0.37	0.02	
	6/7	0.33	0.11	0.01	0.33		0.30	0.10	0.40	—0.07	
	Average				0.36	0.017				—0.02	—0.001
III	J.E.										
	6/2	0.40	0.01	0.01	0.40		0.49	0.10	0.59	—0.19	
	6/7	0.34	0.01	0.01	0.34		0.11	0.08	0.19	0.15	
	Average				0.36	0.018				—0.02	—0.001
II	J.E.										
	3/1	0.38	0.00	0.00	0.38	0.019	0.27	0.09	0.36	0.02	0.001
II	F.V.										
	2/25	0.40	0.00	0.00	0.40		0.62	0.07	0.69	—0.29	
	3/1	0.38	0.00	0.00	0.38		0.12	0.08	0.20	0.18	
	Average				0.39	0.020				—0.06	—0.003
II	G.E.										
	2/25	0.38	0.00	0.00	0.38		0.32	0.07	0.39	0.01	
	3/1	0.33	0.00	0.00	0.33		0.21	0.06	0.27	0.06	
	Average				0.35	0.021				0.03	0.001
III	G.E.										
	5/12	0.41	0.02	0.01	0.42		0.34	0.11	0.45	—0.03	
	5/17	0.40	0.02	0.01	0.41		0.28	0.09	0.37	0.04	
	Average				0.41	0.023				0.01	0.000
I	G.E.										
	2/4	0.36	0.00	0.01[2]	0.35		0.17	0.10	0.27	0.08	
	2/9	0.41	0.00	0.01[2]	0.40		0.34	0.09	0.43	—0.03	
	Average				0.38	0.023				0.03	0.002
I	J.E.										
	2/4	0.48	0.00	0.01[2]	0.47		0.44	0.02	0.46	0.01	
	2/9	0.51	0.00	0.01[2]	0.50		0.47	0.03	0.50	0.00	
	Average				0.49	0.024				0.01	0.000
I	F.V.										
	2/4	0.51	0.00	0.01[2]	0.50		0.29	0.01	0.30	0.20	
	2/9	0.54	0.00	0.01[2]	0.53		0.52	0.10	0.62	—0.09	
	Average				0.51	0.028				0.06	0.003
III	F.V.										
	5/12	0.65	0.00	0.01	0.64		0.81	0.10	0.91	—0.27	
	5/17	0.55	0.00	0.01	0.54		0.36	0.16	0.52	0.02	
	Average				0.59	0.029				—0.12	—0.006
III	F.V.[1]										
	4/21	0.54	0.00	0.00	0.54		0.27	0.10	0.37	0.17	
	4/26	0.63	0.00	0.00	0.63		0.67	0.12	0.79	—0.16	
	Average				0.59	0.029				0.01	0.000
III	G.E.										
	4/21	0.48	0.01	0.00	0.49		0.32	0.09	0.41	0.08	
	4/26	0.52	0.01	0.00	0.53		0.48	0.05	0.53	0.00	
	Average				0.51	0.029				0.04	0.002
III	J.E.										
	4/21	0.56	0.01	0.00	0.57		0.28	0.12	0.40	0.17	
	4/26	0.60	0.01	0.00	0.61		0.45	0.08	0.53	0.08	
	Average				0.59	0.029				0.12	0.005
III	J.E.										
	5/12	0.68	0.02	0.01	0.69		0.63	0.07	0.70	—0.01	
	5/17	0.58	0.02	0.01	0.59		0.54	0.07	0.61	—0.02	
	Average				0.64	0.030				—0.02	—0.001

[1] Slight regurgitation not analyzed.

[2] Estimated values.

their spectra with those produced by solutions of known concentration.

Neither the fecal nor the urinary excretion of aluminum bore any relation to the amount of aluminum ingested. Some aluminum was present in all the urines, although the greater proportion of the ingested aluminum appeared in the feces.

Fifty per cent of the balances were positive and 50% were negative. Sixty per cent of all of these fell within the ±10% error as determined for the method used in this study. The amount of aluminum retained in the remaining 40% of the balances suggested that aluminum is not an essential constituent of the diets of preschool age children.

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LITERATURE CITED

Beal, C. D., R. B. Unangst, H. Wigman and G. J. Cox 1932 II. Aluminum content of foodstuffs cooked in aluminum and in glass. Ind. Eng. Chem., vol 24, p. 405.

Cox, G. J., M. I. Dodds, H. B. Wigman and F. J. Murphy 1931 Effects of high doses of aluminum and iron on phosphorus metabolism. Proc. Am. Soc. Biol. Chem., vol. 92, p. xi.

Cox, G. J., E. W. Schwartz, R. M. Hann, R. B. Unangst and J. L. Neal 1932 Occurrence and determination of aluminum in foods. Ind. Eng. Chem., vol. 24, p. 403.

Deobald, H. J., and C. A. Elvehjem 1935 Effect of feeding high amount of soluble iron and aluminum salts. Am. J. Physiol., vol. 111, p 118.

Dee, T., and O. S. Rask 1931 Spectrographic determination of aluminum in biological ashes. J. Ind. Eng. Chem. Anal. Ed., vol. 3, p. 39.

Eveleth, D. F., and V. C. Myers 1936 Studies on aluminum. I. A critical study of quantitative colorimetric methods for aluminum in biological materials. J. Biol. Chem., vol. 113, p. 449.

Flynn, F. B., and I. M. Inouye 1928 Metals in foods. J. Am. Med. Assoc., vol. 90, p. 1010.

Harrison, G. H. 1936 Practical possibilities in spectrographic analysis. Metals and Alloys, vol. 7, p. 291.

Kahlenberg, L., and J. C. Closs 1929 Presence of aluminum in plant and animal matter. J. Biol. Chem., vol. 83, p. 261.

LAMB, E. W. 1935 Spectrographic determination of impurities in commercial cadmium. Proc. Am. Soc. Testing Materials, Part II, vol. 35, p. 71.

LAMPITT, L. H., AND N. D. SYLVESTER 1932 Determination of small amounts of aluminum in food. Analyst, vol. 57, p. 418.

LEWIS, S. J. 1931 CXXII. The assimilation of aluminum by the human system. Biochem. J., vol. 25, p. 2162.

MACKENZIE, K. 1930 Biochemistry of aluminum. I. Circulation and absorption of aluminum in the pig. Biochem. J., vol. 24, p. 1433.

——— 1931 Biochemistry of aluminum. II. Excretion and absorption of aluminum in the rat. Biochem. J., vol. 25, p. 287.

MCCOLLUM, E. V., O. S. RASK AND E. BECKER 1928 A study of the possible role of aluminum compounds in animal and plant tissue. J. Biol. Chem., vol. 77, p. 753.

MYERS, V. C., AND J. W. MULL 1928 Influence of the administration of aluminum upon the aluminum content of the tissues and upon the growth and reproduction of rats. J. Biol. Chem., vol. 78, p. 605.

REFEREE BOARD OF CONSULTING SCIENTIFIC EXPERTS (Ira Remsen, chairman) 1914 Aluminum in foods. U. S. Dept. Agr. Bull., no. 103.

SCHWARTZ, E. W., G. J. COX, R. B. UNANGST, F. J. MURPHY AND H. B. WIGMAN 1933 Extent of retention of aluminum. J. Am. Med. Assoc., vol. 101, p. 1722.

SCOULAR, FLORENCE I. 1938 A quantitative study by means of spectrographic analysis of copper in nutrition. J. Nutrition, vol. 16, p. 437.

SCOULAR, FLORENCE I. 1939 A quantitative study, by means of spectrographic analysis, of zinc in nutrition. J. Nutrition, vol. 17, p. 103.

SMITH, E. E. 1928 Aluminum compounds in foods. Paul B. Hoeber, Inc., New York.

TOCCO, L., AND M. MULAS 1935 Sulla tossicita dell'-alluminio. II. La palvers di aluminio. Arch. internat. Pharmocodyn., vol. 51, p. 159. (Int. Pharmocal, therap. R. U. Sassari.)

UNDERHILL, F. P., AND F. I. PETERMAN 1929 Studies in the metabolism of aluminum. III. Absorption and excretion in the normal man. Am. J. Physiol., vol. 90, p. 40.

UNDERHILL, F. P., F. I. PETERMAN, E. G. GROSS AND A. C. KRAUSE 1929 Studies in the metabolism of aluminum. VII. Aluminum content of some fresh foods. Am. J. Physiol., vol. 90, p. 72.

WINTER, O. B., AND C. D. BIRD 1929 Determination of aluminum in plants. II. In plant materials. J. Am. Chem. Soc., vol. 51, p. 2964.

THE NEED FOR MANGANESE IN THE BONE DEVELOPMENT OF THE CHICK

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ONE FIGURE

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Manganese was found by Wilgus, Norris and Heuser ('36, '37 a) to be markedly effective in preventing the incidence of perosis in chicks. The symptoms of perosis generally recognized are enlargement of the tibio-metatarsal joint, twisting and bending of the distal end of the tibia and of the proximal end of the tarso-metatarsus and slipping of the gastrocnemius tendon from its condyles.

In addition to these symptoms Herner and Robinson ('32) and Wilgus, Norris and Heuser ('37 b) reported that frequently the leg bones of chicks fed perosis-producing diets are thickened and shortened. In some instances the thickening and shortening of the leg bones was observed unaccompanied by other symptoms of perosis. On the other hand, Titus ('32) stated that in rickets the tarso-metatarsi and to some extent the tibiae are shortened and thickened whereas in perosis these bones are not affected in this manner. Also Holmes, Pigott and Moore ('33) measured the tibiae of normal chicks and of chicks afflicted with slipped tendon fed the same diet and concluded that the results obtained did not reveal any significant differences.

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[1] On sabbatical leave of absence from the Oklahoma Agricultural and Mechanical College, 1937–1938.

[2] On sabbatical leave of absence from the Oklahoma Agricultural Experiment Station, 1936–1937.

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Later Gallup and Norris ('38) made a preliminary report of the results of measuring the leg bones of chicks afflicted with perosis as a result of being fed a low-manganese diet and of normal chicks fed an adequate diet. They found that the chicks suffering from perosis had leg bones 7 to 8% shorter than those of the normal chicks of the same weight, sex and age. These workers also reported that a large percentage of the chicks hatched from eggs of low-manganese content have short leg bones. This observation is in agreement with a report by Lyons and Insko ('37) that chondrodystrophic embryos characterized by shortened and thickened legs, short wings and other deformities were produced from the eggs of hens fed a low-managanese diet. The difference between the length of the leg and wing bones of chondrodystrophic embryos and those of normal embryos was especially striking.

Although the results previously discussed are not in complete agreement it appeared probable that manganese plays a more important role in the bone development of the chick than merely the prevention of the anatomical deformities of the legs generally recognized as symptoms of perosis. Hence, a detailed study was initiated to determine the significance of the difference in bone development between chicks afflicted with perosis and normal chicks observed by Gallup and Norris ('38).

EXPERIMENTAL PROCEDURE AND RESULTS

Two lots of 100-day-old Rhode Island Red chicks were fed low-manganese (5.5 p.p.m.) and adequate-manganese (100 p.p.m.) diets respectively. The basal diet used was a modification of that used by Wilgus, Norris and Heuser ('37 b). The substitution of 20% of degerminated yellow cornmeal for a like amount of ground yellow corn and the replacement of fish meal with meat scrap were the principal changes in the diet.

At the end of 2 weeks and weekly thereafter for 4 weeks chicks approximately representative of the low-manganese lot as determined by the weight distribution curve were paired with representative chicks of the same sex and weight from the adequate-manganese lot. The chicks were killed and the femurs dissected out with cartilage caps in place. Then the bones were measured, weighed individually and the amount of moisture, fat and ash determined.

The results presented in table 1 show that at 2 weeks of age the femurs of the Rhode Island Red chicks fed the low-manganese diet were approximately 1.8% shorter than those of the chicks of the same age, sex and approximately the same weight fed the adequate-manganese diet. At 3 weeks of age the difference had increased to 7.5% and at 4 weeks to 8.7%. These results when analyzed by Student's method were found to be highly significant. Beyond 4 weeks of age no direct comparisons of bone length were made, since manganese deficiency was beginning to retard the growth rate seriously which would make it necessary to pair the more rapidly growing chicks of the low-manganese lot with the slower growing chicks of the adequate-manganese lot.

TABLE 1

Effect of manganese on the development of the femurs of chicks[1]

MANGANESE IN DIET	CHICKS PER GROUP	AVERAGE WEIGHT	AVERAGE LENGTH OF FEMURS	DIFFERENCE	ODDS THAT DIFFERENCE IS SIGNIFICANT[2]
p.p.m.		*gm*	*mm.*	*%*	
		Age 2 weeks			
5.5	11	120.0	33.8	1.8	17 : 1
100	11	118.6	34.4		
		Age 3 weeks			
5.5	11	172.5	38.0	7.5	713 : 1
100	11	173.0	41.1		
		Age 4 weeks			
5.5	9	222.8	42.3	8.7	3332 : 1
100	9	222.0	46.4		

[1] Rhode Island Red.

[2] Student's method.

It was decided, therefore, to use chicks of different age but of the same weight and sex in making a series of comparisons. The results presented in table 2 show that, even though the low-manganese chicks were older than those with which they were compared, their femurs were 5.7% shorter, a difference which proved to be highly significant statistically.

In making the same type of comparison in another experiment with the exception that growth was retarded by a deficiency of riboflavin instead of manganese it was found that the older retarded chicks had the longer bones in spite of the fact that their weights were approximately the same as those of the younger, normal chicks. It appears, therefore, that the shortening of the bones in the low-manganese lot is not connected with the growth-retarding effect of a deficiency of manganese.

TABLE 2

Effect of manganese on the development of the femurs of chicks[1] of the same weight but of different age

MANGANESE IN DIET	CHICKS PER GROUP	AGE OF CHICKS	AVERAGE WEIGHT	AVERAGE LENGTH OF FEMURS	DIFFERENCE	ODDS THAT DIFFERENCE IS SIGNIFICANT[2]
p p m		*wk*	*gm.*	*mm.*	%	
5.5	15	7	529.4	54.4	5.7	311 : 1
100	15	6	520.5	57.7		

[1] Rhode Island Red.

[2] Student's method.

Since chicks fed a diet deficient in manganese are consistently retarded in growth, it was thought advisable to compare the bone length of two lots of retarded chicks, one of which was retarded in growth by a deficiency of manganese, the other by injection of excess quantities. The results of this study are presented in table 3. They show that the length of the femurs of the chicks, the growth of which was retarded by means of a low-manganese diet, was 8.3% shorter than that of the chicks whose growth was retarded by the injection of excess manganese. This difference by Student's method was found to be highly significant.

In the foregoing the comparisons of bone length were made between paired chicks. Since the chicks selected were not an absolutely representative sample of the lot, it was felt desirable to use some method which would give a more complete comparison between the two lots as a whole. By plotting upon logarithmic paper the average length of the femurs against the average body weight of chicks from representative samples of the two lots curves were obtained the slopes of which gave the ratio of the increase in bone length to increase in body weight. The slope of the curve of the low-manganese lot was found to be less than that of the high-manganese lot. This showed that the retardation in growth of the bones in length is less than the retardation in increase in body weight of the chicks.

TABLE 3

Effect of manganese on the development of the femurs of retarded chicks[1]

RETARDED BY	CHICKS PER GROUP	AVERAGE WEIGHT	AVERAGE LENGTH OF FEMURS	DIFFERENCE	ODDS THAT DIFFERENCE IS SIGNIFICANT[2]
		gm	*mm*	%	
Low-manganese diet	7	365.0	44.3		
Injection of excess manganese	7	356.5	48.3	8.3	330 : 1

[1] Rhode Island Red, age 6 weeks.

[2] Student's method.

The bones of the wings are affected by a deficiency of manganese the same as the bones of the legs but not quite to the same extent. At 3 weeks of age it was found that the average shortening of the femurs of chicks fed a low-manganese diet was 8.3%, the tibiae 9.5% and the tarsometatarsi 5.7% whereas the average shortening of the humeri was 6.8%, the ulnae 6.7% and the metacarpi 2.2%. It was observed also that the spinal column of day-old chicks of hens fed a low-manganese diet is frequently shorter than that of day-old chicks of the same weight of hens fed an adequate diet. As a result of the effect of a deficiency of manganese upon bone development, the body conformation of the chick is altered, resulting in a stockier individual with a deeper body. In figure 1 are presented illustrations of the effect of a deficiency of manganese upon the development of the bones of the legs and wings.

The results of twenty comparisons of the leg bones of White Leghorn chicks 4 weeks old agree essentially with those obtained in the studies on Rhode Island Red chicks. The tibiae of the White Leghorn chicks on the low-manganese diet averaged 2.6% shorter than those of their adequately fed controls, and the tarso-metatarsi averaged 1.4% shorter. The smaller differences in bone length shown by this breed agree with the observation that White Leghorn chicks are less susceptible to perosis than the American breeds. Recent experiments showed that the manganese requirement of White Leghorn chicks is relatively low (Insko, Lyons and Martin, '38; Gallup and Norris, '39 a).

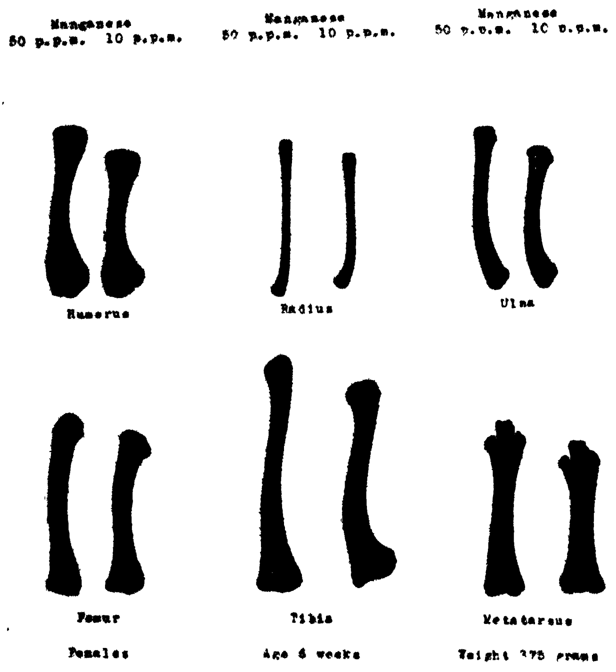

Fig. 1 Effect of manganese on the development of the leg and wing bones of chicks.

The essentialness of manganese for the normal development of bone in the chick is further emphasized by the results of measurements of the leg bones of day-old chicks hatched from the eggs of hens fed low-, medium- and high-manganese diets. The results are presented in table 4. They show that a manganese deficiency in the diet of hens results in the development of short leg bones in the chicks hatched from their eggs.

The chicks hatched from the eggs of two hens in the second low-manganese lot were exceptions, however, to this statement as they possessed leg bones of normal length. This is explained by the fact that upon analysis the eggs of these hens were found to contain 0.063 mg. of manganese per 100 gm. of dry egg yolk, presumably because of a more efficient transfer of manganese from the diet to the egg, while those of the other hens in this lot contained only 0.039 mg. of manganese per 100 gm. Gallup and Norris ('39 b) and others showed that hens fed a low-manganese diet generally lay eggs of low-manganese content.

TABLE 4

Effect of the manganese content of the maternal diet upon embryonic bone development

MANGANESE IN MATERNAL DIET	DAY-OLD CHICKS[1] PER GROUP	AVERAGE BONE LENGTH		DIFFERENCE	
		TIBIA	METATARSUS	TIBIA	METATARSUS
p.p m.		*mm.*	*mm.*	%	%
13	16	26.0	18.7	12.5	14.2
53	6	29.7	21.8		
13	12	27.4	19.7	13.1	15.1
200	25	31.5	23.2		

[1] New Hampshire.

The retardation in bone length in chicks fed a low-manganese diet cannot be explained on the basis of an increased water content in the body which would make it appear that relatively the body was developing faster than the bones. A study of the gross composition of chicks fed a low-manganese diet and of chicks fed an adequate-manganese diet at 4 weeks of age showed that the body composition of both groups of chicks was approximately the same, the former containing 69.3% water, 5.81% fat and 3.90% ash and the latter containing 69.3% water, 4.91% fat and 3.93% ash.

In table 5 are presented the results of a study of the ash content of the femurs of representative chicks from the low-manganese and adequate-manganese lots. The ash content of the femurs of the low-manganese chicks decreased slightly with increase in age whereas that of the adequate-manganese chicks remained approximately constant. The differences in the ash content at 5 and 6 weeks of age are highly significant statistically. They are not due to difference in size of chick, since the bone-ash content of the low-manganese chicks at 6 weeks of age was less than that of the adequate-manganese chicks at 3 weeks of age at which time the former chicks weighed more than the latter. The results obtained in this study on the bone-ash content of chicks fed a low-manganese diet do not agree with those of other workers (Hogan, Shrewsbury and Kempster, '28; Hunter and Funk, '30; Hall and King, '31 and others) whose studies on the ash content of the

TABLE 5

Effect of manganese on the ash content of the femurs of chicks[1]

MANGANESE IN DIET	CHICKS PER GROUP	AVERAGE WEIGHT	AVERAGE WEIGHT OF FEMURS	ASH IN FAT FREE DRY BONES	t VALUE[2]
p.p.m.		*gm.*	*gm*	%	
		Age 2	weeks		
5.5	15	120.2	0.727	40.66	2.149
100	15	122.1	0.742	42.01	
		Age 3	weeks		
5.5	15	166.6	1.30	42.80	0.987
100	15	179.7	1.37	43.42	
		Age 5	weeks		
5.5	15	268.0	2.10	41.65	4.984
100	15	381.2	2.85	44.78	
		Age 6	weeks		
5.5	15	300.7	2.74	40.45	6.259
100	15	448.0	3.77	44.35	

[1] Rhode Island Red.

[2] For odds of 19 to 1 t must have a value of 1.990. For odds of 99 to 1 t must have a value of 2.638.

bones of chicks afflicted with perosis failed to reveal any marked variation from the normal.

The reduction in bone ash in the low-manganese lot was not due to rickets in the generally accepted meaning of the term. The diet fed the chicks in the low-manganese lot as well as that fed the adequate-manganese lot contained approximately 1.85% of calcium and 0.85% of phosphorus, amounts according to Wilgus ('31) in excess of the minimum requirements for normal bone formation. These diets also contained approximately 65 A.O.A.C. units of vitamin D per 100 gm., three times the minimum amount found necessary to prevent rickets by Murphy, Hunter and Knandel ('36). The serum calcium[3] of the chicks fed these diets was within the normal range of 10 to 12 mg. per 100 cc. of blood serum. In studies on the relation of vitamin D to the manganese requirement[4] it was observed that in chicks fed the same low-manganese diet plus 20 A.O.A.C. units of vitamin D per 100 gm. the calcification of the bones was normal upon examination in accordance with the procedure of McCollum, Simmonds, Shipley and Park ('22). Also no difference in calcification was observed by Gallup and Norris ('38) as a result of x-ray examination of the leg bones of chicks fed a low-manganese diet and those of chicks fed an adequate diet. For these reasons it appears highly improbable that manganese favorably influences vitamin D utilization. Rather it has been found by Caskey and Norris ('38) that the effectiveness of manganese in preventing perosis in chicks fed a high-calcium, high-phosphorus diet is decreased when an adequate amount of vitamin D is supplied.

In view of these considerations the conclusion that manganese along with vitamin D is concerned with calcium and phosphorus metabolism in the chick appears inescapable. This conclusion is supported by the results of Caskey and Norris ('38) showing that the breaking strength of eggs and egg-shell ash (CaO) are directly correlated with the manganese content of the diet fed the hens. It is also supported by

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[3, 4] Unpublished results.

the results of Wiese, Johnson, Elvehjem and Hart ('38) who found that blood and bone phosphatase and ester phosphorus of the blood are depressed in manganese deficiency in the chick. The corollary of this conclusion is that the abnormal shortening of the bones of chicks is probably the direct effect of the upset in calcium and phosphorus metabolism resulting from manganese deficiency.

CONCLUSIONS

A deficiency of manganese in the diet of chicks results in a significant shortening of the bones of the legs and wings as well as a shortening of the spinal column. The shortening of the bones cannot be explained by an alteration in the gross composition of the body, since the body composition of both lots of chicks was found to be approximately the same.

It was also found that the ash content of the bones of chicks fed a low-manganese diet was significantly lower than that of chicks fed an adequate diet. This was not due to the development of a rachitic condition from defective vitamin D utilization, as the low-manganese diet contained excess vitamin D, serum calcium was within the normal range and examination of the leg bones by means of the x-ray and by staining sections with silver nitrate showed calcification to be normal.

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LITERATURE CITED

CASKEY, C. D., AND L. C. NORRIS 1938 Further studies on the role of manganese in poultry nutrition. Poul. Sci., vol. 17, p. 433.

GALLUP, W. D., AND L. C. NORRIS 1938 The essentialness of manganese for the normal development of bone. Science, vol. 87, p. 18.

———— 1939 a The amount of manganese required to prevent perosis in the chick. Poul. Sci., vol. 18, p. 76.

———— 1939 b The effect of a deficiency of manganese in the diet of the hen. Poul. Sci., vol. 18, p. 83.

HALL, G. E., AND E. J. KING 1931 Calcium and phosphorus metabolism in the chicken. Poul. Sci., vol. 10, p. 259.

HERNER, M. C., AND A. D. ROBINSON 1932 A study of leg-bone deformities in growing chicks. Poul. Sci., vol. 11, p. 283.

HOGAN, A. G., C. L. SHREWSBURY AND H. L. KEMPSTER 1928 Rapid growth of chicks on rations of natural foodstuffs. J. Agr. Res., vol. 37, p. 115.

HOLMES, A. D., MADLEINE G. PIGOTT AND W. B. MOORE 1933 Mineral content of tibiae from chicks with slipped tendon. Poul. Sci., vol. 12, p. 356.

HUNTER, J. E., AND E. M. FUNK 1931 The production of 'slipped tendon' in chicks by experimental feeding. Proc. Poul. Sci. Assoc., 22nd ann. meet., p. 45.

INSKO, W. M., JR., M. LYONS AND J. H. MARTIN 1938 The quantitative requirement of the growing chick for manganese. J. Nutrition, vol. 15, p. 621.

LYONS, M., AND W. M. INSKO, JR. 1937 Chondrodystrophy in the chick embryo produced by manganese deficiency in the diet of the hen. Ky. Agr. Exp. Sta. Bull. 371.

MCCOLLUM, E. V., N. SIMMONDS, P. G. SHIPLEY AND E. A. PARK 1922 A delicate biological test for calcium-depositing substances. J. Biol. Chem., vol. 51, p. 41.

MURPHY, R. R., J. E. HUNTER AND H. C. KNANDEL 1936 The vitamin D requirements of growing chicks and laying hens. Penn. Agr. Exp. Sta. Bull. 334.

TITUS, H. W. 1932 Perosis, or deforming leg weakness in the chicken. Poul. Sci., vol. 11, p. 117.

WILGUS, H. S., JR. 1931 The quantitative requirement of the growing chick for calcium and phosphorus. Poul. Sci., vol. 10, p. 107.

WILGUS, H. S., JR., L. C. NORRIS AND G. F. HEUSER 1936 The role of certain inorganic elements in the cause and prevention of perosis. Science, vol. 84, p. 252.

———— 1937 a The role of manganese and certain other trace elements in the prevention of perosis. J. Nutrition, vol. 14, p. 155.

———— 1937 b The effect of various calcium and phosphorus salts on the severity of perosis. Poul. Sci., vol. 16, p. 232.

WIESE, A. C., B. C. JOHNSON, C. A. ELVEHJEM AND E. B. HART 1938 Phosphorus metabolism of chicks afflicted with perosis. Science, vol. 88, p. 383.

CITRIC ACID AND ITS SALTS AS CALCIFYING AGENTS IN RATS

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The recent reviews of Schmidt and Greenberg ('35) and Gubner ('37) have summarized available information concerning calcium and phosphorus metabolism and their relations to rickets. They serve also to bring out the complexity of the problem of bone formation and to point out the conflicting views on how it is brought about.

Reports of early work on the effect of the acid-base value of the diet on rickets have been summarized by Morris, Ford and Graham ('36). Although their own experiments showed no evidence of acidosis as a causal or associated factor in infantile rickets, the earlier studies had shown conflicting results.

Early studies on the use of citric acid milk in preventing or curing infantile rickets also showed conflicting results. Weissenberg ('28) stated that his 'Zitronensäurevollmilch' prevented or cured rickets in the majority of cases, but Hess and Matzner ('24) did not find lemon juice milk effective without the addition of egg yolk.

The importance of citrates in calcification has received rather minor attention until the recent work of Shohl ('37) and Hamilton and Dewar ('37). Shohl showed by histological and x-ray studies that the addition of citric acid-sodium citrate or tartaric acid-sodium tartrate mixtures to rachitogenic diets will render them non-rachitogenic, and Hamilton and Dewar showed by the measurement of the width of the epiphyseal space on x-ray plates that the addition of sodium citrate, citric acid, sodium tartrate, sodium bitartrate or tartaric acid would give normal width to the epiphyseal line. They also secured normal or nearly normal line tests (3.5 or better) on rats fed these substances according to the vitamin D test technic (U. S. Pharmacopeia XI). These two papers differ in that the latter authors found the acids or the salts effective whereas Shohl found only the mixtures effective in producing normal x-ray and histological results.

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Thus we see that the literature contains many contradictions, and in the light of these contradictions the present study of citric acid and citrates as calcifying agents was undertaken.

PLAN OF THE EXPERIMENT

Young albino rats from our own colony weighing 35 to 50 gm. at 21 to 25 days of age were placed in individual cages in a dark room. The animals in both experiments I and II were placed on the Steenbock-Black rachitogenic diet 2965 ('25) for 22 days. They were then given an experimental diet for 10 days and were killed on the eleventh day. The hind legs were dissected from the body, the femurs preserved in 95% ethyl alcohol, the tibias in acetone, for analysis for bone ash and line test respectively. Record of food intakes and body weights were made two to three times weekly throughout the study but no attempt was made to control food intakes. The experimental diets were prepared by adding to 100 gm. rachitogenic diet the following supplements:

Experiment I	*mol.*
1. None (controls)	
2. Citric acid	0.04
3. Sodium citrate	0.04
4. Citric acid +	0.02
sodium citrate	0.02
5. Citric acid +	0.03
sodium citrate	0.02
6. Lactic acid	0.04

Experiment II	*mol.*
1. None (controls)	
2. Potassium citrate	0.04
3. Citric acid +	0.02
potassium citrate	0.02
4. Citric acid +	0.03
potassium citrate	0.02

The technic used in the preparation and reading of the line tests was that of McCollum, Simmonds, Shipley and Park ('22). For the bone ash determinations each pair of femurs and the rat numbers were tied in small pieces of cheese cloth and extracted with ethyl alcohol for 48 hours in a soxlet extraction apparatus. They were then extracted with ether for 24 hours. The details of the method used for weighing and ashing the bones are given by Lachat ('35). The 1 hour heating was used, and the samples were reheated and reweighed as a check for complete ashing.

EXPERIMENTAL RESULTS

The results of these studies are given in table 1. They were separated into experiments I and II because the rats were from different litters, the initial weights of the animals were different and the food intakes of the animals in experiment II were greater both during the depletion and the experimental periods. It is of interest to note, however, that the average gains in weight of the corresponding groups are strikingly similar in spite of these differences.

Although the animals do not show complete agreement between line tests and bone ash values, the following summary may be made from the table of the probable effectiveness as antirachitic agents of the supplements used.

Since calcification in the controls was very poor, evidenced both by line test and bone ash values, the basal diet was truly rachitogenic. The addition of citric acid (0.04 mol. per 100 gm. basal ration) resulted in only limited improvement, more marked in the epiphyseal region than throughout the bone. The odds that the differences in bone ash values found are not due to chance are only 28:1, so that these results are barely significant. Sodium citrate (0.04 mol. per 100 gm. basal ration) and a mixture of equal parts of citric acid and sodium citrate (i.e., 0.02 mol. of each) averaged practically identical results and showed definitely better calcification than the citric acid alone, both by line test and by bone ash values. The odds for the significance of these bone ash values are

TABLE 1
The effect of acid and of acid-salt mixtures on calcification

SUPPLEMENT	LITTER	SEX	WEIGHT AT START OF EXPERIMENT	DEPLETION PERIOD 22 DAYS		EXPERIMENT PERIOD 10 DAYS		LINE TEST	BONE ASH
				Gain in weight	Food intake	Gain in weight	Food intake		
Experiment I									
			gm.	gm.	gm.	gm.	gm.		%
1. None (controls)	1	♀	34	18	121	12	67	R	22.0
	2	♂	46	30	144	18	82	R-1	28.6
	3	♂	40	24	121	6	63	R	23.2
	4	♀	38	14	97	11	54	R	26.3
	4	♀	40	16	110	4	50	R	24.0
		Av.	40	20	119	10	63	R	24.8
2. Citric acid	1	♂	38	14	98	10	56	2	29.6
0.04 mol. in	2	♀	43	23	122	8	68	2.5	30.0
100 gm. diet	3	♂	36	17	114	11	64	1.5	24.0
	3	♀	34	22	120	12	66	1	27.7
	4	♂	38	22	117	12	69	3	33.8
		Av.	38	20	114	11	65	2.0	29.0
3. Sodium citrate	1	♀	34	12	99	4	56	3.5	34.3
0.04 mol. in	2	♂	44	28	135	4	60	4	35.4
100 gm. diet	3	♂	42	20	105	10	63	3	31.7
	3	♀	36	22	116	6	57	2.5	32.5
	4	♂	43	19	116	2	49	4	33.0
		Av.	40	20	114	5	57	3.4	33.4
4. Citric acid	1	♀	34	20	114	2	44	4	32.0
0.02 mol. plus	2	♂	40	23	123	11	65	3.5	32.2
sodium citrate	2	♀	42	23	132	9	60	3.5	37.3
0.02 mol. in	3	♂	40	22	123	10	65	3	33.3
100 gm. diet	4	♂	35	19	113	11	54	3	36.4
	4	♀	35	17	103	14	50	3.5	29.6
		Av.	38	21	118	10	56	3.4	33.5
5. Citric acid	1	♂	35	19	106	8	47	3.5	28.9
0.03 mol. plus	2	♂	42	16	112	8	63	4	37.9
sodium citrate	2	♀	40	20	102	8	64	4	39.6
0.02 mol. in	3	♂	40	22	123	10	63	3.5	32.4
100 gm. diet	4	♀	35	23	110	5	49	4	38.5
		Av.	38	20	111	8	57	3.8	35.5
6. Lactic acid	1	♂	36	20	108	10	47	1	22.5
0.04 mol. in	1	♀	38	24	125	8	54	1.5	28.4
100 gm. diet	2	♂	40	20	113	2	58	2.5	34.0
	3	♀	38	22	127	4	55	R	25.3
	4	♀	34	18	103	6	52	1	25.0
		Av.	37	21	115	6	53	1.2	27.0
Experiment II									
1. None (controls)	5	♂	42	20	119	10	92	R	25.0
	6	♀	48	22	162	22	96	R	29.2
	6	♂	45	29	179	18	100	R	26.0
	7	♀	50	20	159	12	82	R	21.0
		Av.	46	23	155	16	93	R	25.3
2. Potassium citrate	5	♂	43	20	123	1	77	3	35.1
0.04 mol. in	5	♂	48	24	155	4	105	3.5	39.9
100 gm. diet	6	♀	58	20	179	12	94	3.5	41.0
	7	♂	52	18	152	10	93	4	38.6
	7	♀	43	19	159	8	78	3	36.1
		Av.	49	20	154	7	89	3.4	38.1
3. Citric acid	5	♀	42	19	134	7	98	3	37.8
0.02 mol. plus	5	♂	50	21	146	9	104	3.5	41.4
potassium citrate	6	♂	48	24	169	18	110	4	46.1
0.02 mol. in	6	♀	46	28	172	14	100	3	38.6
100 gm. diet	7	♂	53	19	157	13	106	4	41.4
		Av.	48	22	156	12	104	3.5	41.1
4. Citric acid	5[1]	♂	46[1]	22[1]	133[1]	—8[1]	51[1]	4[1]	31.4[1]
0.03 mol. plus	5	♀	42	24	145	2	93	3	39.5
potassium citrate	6	♂	52	23	166	13	95	4	43.0
0.02 mol. in	7	♂	50	22	165	10	106	4	36.8
100 gm. diet	7	♀	50	20	160	10	85	4	38.9
		Av.	49	22	159	9	95	3.8	39.6

[1] Omitted in average since rat lost 8 gm. and did not eat at least 2 gm. every day during the experimental period (see U.S. Pharmacopeia XI).

3332:1 or over so that they are truly significant. The use of 0.03 mol. citric acid plus 0.02 mol. sodium citrate, equivalent to the proportion of citric acid-sodium citrate used by Shohl, did not significantly increase the improvement in calcification over the values on the lower concentration of citrates when at least 0.02 mol. of the citrate was given as salt. Therefore no tests were made by us of the higher concentration of citrate as either citric acid or sodium citrate alone. The odds were 57:1 that these values were more significant than the values found when citric acid alone was used. Lactic acid at 0.04 mol. level showed only slight improvement as measured by either line test or bone ash, and the results were not statistically significant.

In experiment II the addition of potassium citrate (0.04 mol. per 100 gm. basal ration), citric acid 0.02 mol. plus potassium citrate 0.02 mol., and citric acid 0.03 mol. plus potassium citrate 0.02 mol. all gave very satisfactory results; there were no line tests below 3 + cure and no bone ash values below 35%. The slightly higher line test in the citric acid 0.03 mol. plus potassium citrate 0.02 mol. did not seem significant and the bone ash values were somewhat lower. The odds for the significance of the improvement in bone ash values by the addition of potassium citrate or citric acid—potassium citrate mixtures were all beyond the limits of table IX in Love's text ('37), but the ratios were > 3332:1 for the first two supplements and > 1999:1 when the higher concentration of citric acid was used. The differences in the mean values for these three supplements are not statistically signicant.

The results of experiments I and II are not entirely comparable due to litter differences and possibly to differences in initial weights and food intakes, but statistical analysis of the values has shown that potassium citrate was more effective either alone or in combination with equal parts of citric acid than corresponding amounts of sodium citrate. The odds were 249:1 that the improvement was not due to chance using the values for potassium and sodium citrates alone and 712:1

using the mixtures of 0.02 mol. of acid and salt. The differences in values for 0.03 mol. acid plus 0.02 mol. salt were not significant, probably due to the wide variation found among the animals on the citric acid-sodium citrate supplement.

DISCUSSION

Examination of the data presented will show that the variation in food intake among the animals given any specific supplement was greater than the variation in food intake among the animals on different supplements in that experiment. Also there is no evident correlation between the bone ash values and sex, gain in weight or food intake. There seems to be a litter difference, e.g., it will be noted that in experiment I the animal showing the highest bone ash value in each group is from litter 2, except the citric acid 0.04 mol. supplement; in experiment II the animal showing the highest bone ash value in every group is from litter 6; and seven out of the eight animals from each of these litters show bone ash values above the average for their specific supplement.

Before discussing the line test and bone ash results on the different supplements in these experiments, attention should be called to the results on bone ash values on rachitogenic diets reported by Zucker ('36). He found a mean value of 30% ash for rachitic rats at 3 weeks of age, the actual values varying with season, rat strain, etc. Using the 10-day curative experiments used in vitamin D assays (U.S.P. technic) 2 + cures gave a bone ash mean value of 34%. Using fifty or one hundred times the dose required to produce the 2 + cure did not raise the value above 40% although a 4 + cure was found by line test. He also found that the variations in bone ash values increase the further they were removed from the normal. The results of our experiments were comparable to his, mean values of 24.8 to 25.3% were found for control rats, 29% for a 2 + cure, and from 33.4 to 41.1% for 3 to 4 + cure. Only five rats from any group showed values over 40%.

Attention might be called to the line test and corresponding bone ash values given by Shohl ('37). His table 2 shows that with diets S and K, slightly basic basal diets, the addition of the citric acid plus sodium citrate cured the rickets (by histological and x-ray diagnosis) but materially lowered the bone ash values, in diet S by 4%, in diet K by 9%. In the slightly acid diets C and D the 'rickets' was cured with practically no change in bone ash values. Again in table 4 the feeding of lactates gave a slightly better bone ash than the feeding of tartrates, but his decision of cure for the tartrates and no cure for the lactates was again based on the histological and x-ray diagnosis. It would seem that the level of bone ash is more clearly diagnostic of cure than change in the calcification in the epiphyseal line, since improvement in bone ash indicates improved calcification throughout the bone and 'cure of rickets' with continuance of osteoporosis does not materially improve the condition of the animal. Thus from his bone ash values in table 4 the citrates alone have produced normal bones, and in table 2 the bone ash values when citrates were fed were normal only in the case of 8:1 or 4:1 Ca:P ration on diets Y and St.

Our results agree in part with those of Shohl in that lactic acid did not significantly increase the calcification of the femur, and that citric acid produced less significant results alone than when fed as citric acid-sodium citrate mixtures, but they disagree in the amount of citrate ion required and in the fact that in our experiments sodium citrate produced as effective calcification as the citric acid-sodium citrate mixtures. Hamilton and Dewar ('37) reported no bone ash values in their paper, so that the results are not really comparable to ours. However, it might be mentioned that on the basis of line tests and x-ray measurements they found citric acid and sodium citrate about equally effective in producing cures, whereas we found a definite superiority of the salt on the basis of bone ash values. Results on the use of potassium citrate have not been previously reported.

No attempt has been made to explain where or how the citrate acts to increase the calcification, nor why the potassium salt is more effective than the sodium salt. pH studies on the intestinal tracts of these rats indicated that variations there were due more to individual differences than to differences in diet. Obviously there is much more work to be done on this problem, but since circumstances prevent further work on it by either of us at this time, it seemed advisable to report the progress that has been made.

SUMMARY AND CONCLUSIONS

1. The diet used was the Steenbock-Black rachitogenic diet 2965. To this was added lactic acid or citric acid and/or its salts primarily at the level of 0.04 mol. of supplement per 100 gm. basal ration.
2. The increase in bone ash values for the various supplements showed approximately the following averages: lactic acid 2%, citric acid 4%, sodium citrate 9%, citric acid-sodium citrate mixtures 9 to 11%, potassium citrate 13%, citric acid-potassium citrate mixtures 14 to 16%.
3. The effectiveness of citrates in improving the calcification in rats on a rachitogenic diet was confirmed, but the need of citric acid—citrate mixtures for the improvement was not confirmed.
4. The greater effectiveness of potassium citrate as calcifying agent was indicated.
5. The calcifying effect of citrates appeared to be unrelated to sex, gain in weight or food intake.

It is a pleasure to acknowledge the kindness of Dr. F. C. Koch of The University of Chicago in allowing us to use the facilities of his laboratories for the bone ash determinations.

LITERATURE CITED

GUBNER, R. 1937 The metabolic background of rickets. New Eng. J. of Med., vol. 216, p. 879.

HAMILTON, B., AND M. M. DEWAR 1937 Effect of citrate and tartrate on experimental rickets. Am. J. Dis. Children, vol. 54, p. 548.

HESS, A. F., AND M. J. MATZNER 1924 Value of milk acidified with lemon juice; its combination with egg yolk to add the antirachitic factor. J. Am. Med. Assoc., vol. 82, p. 1604.

LACHAT, L. L. 1935 Report on technic and details of biological methods, vitamin D carriers. J. Assoc. Off. Agr. Chem., vol. 18, p. 357.

LOVE, H. H. 1937 Application of statistical methods to agricultural research, The Commercial Press, Limited, Shanghai.

MCCOLLUM, E. V., N. SIMMONDS, P. G. SHIPLEY AND E. A. PARK 1922 Studies on experimental rickets: a delicate biologic test for calcium depositing substances. J. Biol. Chem., vol. 51, p. 41.

MORRIS, N., F. J. FORD AND S. GRAHAM 1936 The role of acidosis and phosphate retention in the pathogenesis of rickets and rachitic tetany of infants. Acta Ped., vol. 18, p. 50.

SCHMIDT, C. L. A., AND D. M. GREENBERG 1935 Occurrence, transport and regulation of calcium, magnesium and phosphorus in the animal organism. Physiol. Rev., vol. 15, p. 297.

SHOHL, A. T. 1937 The effect of the acid-base content of the diet upon the production and cure of rickets with special reference to citrates. J. Nutrition, vol. 14, p. 69.

STEENBOCK, H., AND A. BLACK 1925 Fat soluble vitamins XXIII. J. Biol. Chem., vol. 64, p. 263.

WEISSENBERG, H. 1928 Die Zitronensäurevollmilch in ihrer Bedeutung für die Bekämpfung von Saüglingssterblichkeit und Rachitis. Arch. für Kinderh., Bd. 84, S. 200.

ZUCKER, T. F. 1936 Bone ash in prevention and healing of experimental rat rickets. Science, vol. 84, p. 162.

THE UTILIZATION OF THE CALCIUM OF MILK BY PRE-SCHOOL CHILDREN [1, 2]

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Of all the foods commonly used in the American dietary, milk has long been recognized as being the most important source of calcium. Yet, the extent to which milk calcium is available for growth and for the maintenance of the physiological processes of man has received only scant attention. In estimating an individual's dietary requirement for calcium, it is as necessary to know how well he utilizes the calcium of the foods which he eats as it is to know the actual calcium content of those foods. Until this information is available for milk—and for other foods, too—the value of milk as a source of calcium in the dietary must rest on an insecure basis.

A few investigators have attempted to determine the availability of the calcium of milk, but their studies have been comparisons of milk and other calcium-rich foods or of milk and milk products. In those studies fluid milk has been used as the standard of reference, and the data have been reported in terms of comparative values. Thus, a similarity in the availability of the calcium of American cheese and that of

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[2] Aided by a grant from the American Dry Milk Institute.

[3] A portion of the data obtained on the boys was presented by Elizabeth Jensen in partial fulfillment for the degree of master of science at the University of Illinois, June, 1938.

pasteurized milk was reported by Mallon, Johnson and Darby ('32); Kramer, Potter and Gillum ('31) found raw milk and ice cream made with condensed milk to be equally valuable as sources of calcium. Conversely, a slightly superior value was ascribed to the calcium of evaporated milk over that of pasteurized milk by Willard and Blunt ('27). Likewise, raw milk has a greater comparative value than has dried milk or pasteurized milk, according to Kramer, Latzke and Shaw ('28).

The manner in which the above studies were conducted does not permit an interpretation of the data in terms of absolute values for utilization. According to Mitchell and McClure ('37).

> . . . . the proper method of measuring the degree of utilization of minerals in a feed or ration may be more of a problem than is generally realized. The ordinary method of estimating this utilization is to express the retention of the mineral as a percentage of the intake. However, if there is a maintenance requirement for a mineral, it is not represented in the retention as ordinarily computed, so that a comparison of the latter with mineral intake credits the intake with only part of its nutritive effect and hence underrates its utilization. This underrating becomes the more serious the lower the intake of the mineral. The most practical measurement of the utilization of the mineral content of a feed would seem to be that secured at a level of intake of the particular element studied that just suffices (or that just barely does not suffice) to cover the integrated requirements of the animal for it.

The study reported in this paper constitutes an attempt to determine the extent of the utilization of the calcium of milk by pre-school children. A procedure has been adopted which eliminates the effect of a possible maintenance requirement for calcium. This has been accomplished by determining calcium balances at two levels of intake, both of which gave positive balances and therefore were above the maintenance requirement but neither of which was in excess of the minimum requirement for the maximum retention of calcium by the subjects. In computing percentage utilization, use was made of the increment in calcium retention in relation to the increment in intake between the two levels, rather than (as is commonly done) the total retention in relation to the total intake during one period. This procedure is illustrated by the following formula where 'a' represents a period of high intake and 'b' signifies one of lower intake.

$$\frac{\text{Retention for period a} - \text{retention for period b}}{\text{Intake for period a} - \text{intake for period b}} \times 100 = \text{per cent utilization}$$

This formula is advantageous in that it makes possible a differentiation between retention and utilization and provides for a quantitative, absolute measure of the utilization of the calcium in a given substance, e.g., milk. The only assumption made is that the maintenance requirement is not influenced by the change in the quantity of calcium ingested. Obviously, the formula cannot be applied if the substance used to raise the calcium level causes a change in the utilization of the calcium in the basal diet.

EXPERIMENTAL

The data for this publication were taken from a study of the calcium metabolism of five pre-school girls (Outhouse, Kinsman et al., '39) and from a similar study now in progress on six pre-school boys. The ages, weights and heights and the comparisons of these with Grandprey's ('33) modification of Woodbury's standards are given in table 1 for the boys

TABLE 1

The ages, weights and heights of the subjects and the comparisons of these with Grandprey's ('35) standards

SUBJECT	AGE [1]		WEIGHT [1]	HEIGHT [1]	LOCUS OF MEASUREMENTS		
	Years	Months			Weight-Age	Height-Age	Weight-Height
			kg.	*cm.*	%	%	%
R	2	11	16	99	90	Above 90	75
W	4	0	16	100	35	50	75
C	5	1	20	111	90	90	80
G	5	6	20	115	80	85	70
Br	5	11	23	116	Above 90	85	Above 90
Jw	6	0	20	117	75	85	50

[1] Taken at sixteenth week of experiment.

and in the above publication for the girls. All the subjects were maintained on the regimen described in the aforementioned paper. In both studies calcium balances were determined for every 7 days during the entire experimental period. The time intervals, designated in the reports as periods II, III, IV, etc., varied in length from 28 to 56 days. From period to period, changes in the calcium level were brought about by increasing the quantity of the supplement of milk solids.[4] These increments in calcium were planned to give significant differences in retentions and at the same time, to keep the highest level of intake below the minimum requirement for maximum retention. The experimental procedures adopted for the collection of metabolic materials, for sampling and for analysis for calcium were the same for both the boys and the girls.

A slight difference between the two studies existed wherein the levels of calcium intake were concerned. At the lower level, the girls received 370 mg. of calcium daily whereas the boys received 340 mg. For the higher levels, the girls were given 615 mg.; and, since it was found that this quantity was very close to the minimum requirements of the girls, a smaller quantity (555 mg.) was given to the boys. At the subsequent higher levels of calcium intake, additional values for percentage utilization of the calcium in milk were computable for some of the boys due to the fact that the quantity of calcium ingested at the higher levels was not in excess of their minimum requirements. The data for the various periods with reference to the length of the periods, the average daily intakes of calcium and the amount of calcium contributed by milk solids are presented in table 2.

RESULTS

Only those metabolic data which are pertinent to the subject of the utilization and retention of calcium are presented in this paper (table 3). The actual intakes and retentions of calcium by the individual subjects during the various periods are given in terms of the average daily quantities involved. From the data for retentions, it is apparent that during period IV the 880 mg. level of calcium intake for the girls was in excess of their minimum requirement. For the boys, the level of intake (704 mg. as a daily average) during this period did not give maximum retentions as is indicated by the greater retentions during the subsequent period of higher intake.

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[4] Skim milk powder the calcium content of the various lots of which ranged from 123 to 135 mg. per gram.

TABLE 2

Intakes of calcium, amounts of calcium contributed by milk solids and total calcium retentions during the various periods of the experiment

PERIOD		CALCIUM INTAKE (AVERAGE DAILY)		CALCIUM RETENTIONS (AVERAGE DAILY)
Number	Length	Total	Contributed by milk solids	
		Girls		
	days	*mg*	*mg*	*mg.*
1	77	1815	258	126
2	56	370	258	76
3	35	615	516	122
4	28	880	774	112
		Boys		
1	91	1600	1518	140
2	42	340	231	56
3	35	555	444	100
4	28	704	590	125

Values for the percentage utilization of the calcium of milk have been calculated by the formula presented in the introduction of this paper, using the intake and retention values of periods II and III for the girls and of periods II and III and periods II and IV for the boys. The single values obtained for girls B, M, J, P and S were 18, 14, 22, 25 and 16%, respectively; they averaged 19%. The mean values for boys R, W, C, G, Br and Jw were 18, 21, 20, 13, 19 and 27%, respectively, with a mean value of 20%. The mean value for the eleven subjects was 19% with a standard deviation of 4.5 and a coefficient of variation of 23%. The maximum variation of the values for the subjects was 37% above and 34%

TABLE 3

Total intake and retention of calcium and the utilization of calcium of milk by pre-school children

SUBJECT	INTAKE—AVERAGE DAILY DURING PERIODS				RETENTION								UTILIZATION—COMPUTED FROM DATA FOR PERIODS		
					Average daily during periods				Relation to intake during periods						
	II	III	IV	V	II	III	IV	V	II	III	IV	Average	II & III	II & IV	Average
								Girls							
	mg	mg.	mg.	mg	mg	mg.	mg	mg.	%	%	%	%	%	%	%
B	357	604	862		72	117	85		20.2	19.4		19.8	18.2		
M	357	604	865		50	84	108		14.0	13.9		13.9	13.8		
J	378	615	900		94	145	118		24.8	23.5		24.1	21.5		
P	371	617	875		80	142	154		21.6	23.0		22.3	25.2		
S	384	640	892		84	124	94		21.9	19.3		20.6	15.6		
Mean	369	616	879		76	122	112		20.5	19.8		20.1	18.9		
								Boys							
R	328	554	692	891	63	94	143	158	19.2	16.9	20.6	18.9	13.7	21.9	17.8
W	329	548	703	899	68	116	145	183	20.7	21.1	20.6	20.8	21.0	22.1	21.5
C	347	560	712	909	63	81[1]	136	145	18.1	(14.4)[1]	19.1	18.6	(18.4)[1]	20.0	20.0
G	343	567	720	915	53	83	98	126	15.4	14.6	13.6	14.5	13.4	11.9	12.7
Br	344	556	699	904	60	106	119	179	17.4	19.0	17.0	17.8	21.7	16.6	19.1
Jw	345	545	697	905	64	119	110[1]	135	18.6	21.8	(15.7)[1]	20.2	27.5	(13.2)[1]	27.5
Mean	339	555	704	904	62	103	125	154	18.3	18.0	18.0	18.5	19.5	18.5	19.8
								Girls and Boys							
Mean									19.2	19.2		19.2			19.3

[1] Data not included in calculations since retentions were obviously too low.

below the mean. This variation may appear to be great, yet from a biological standpoint the figures are all of the same order of magnitude and indicate poor utilization of the calcium of milk by pre-school children.

The data for retention of calcium expressed as the percentage of the intake (i.e., percentage retention) for the girls for periods II and III and for the boys for periods II, III and IV are also given in table 3. For girls B, M, J, P and S, the average percentage retentions for periods II and III were 20, 14, 24, 22 and 20, respectively, averaging 20%. For boys R, W, C, G, Br and Jw the average values for periods II, III and IV were 19, 21, 19, 15, 18 and 20, respectively, and averaged 18%. The range and the average of the values for the girls did not differ significantly from those for the boys. The total average for the eleven subjects was 19%.

DISCUSSION

The fact that only from one-fifth to one-fourth of the calcium of milk is utilized and retained by children may seem surprising. However, earlier reports contain data from which the same conclusion could have been drawn. In the Sherman and Hawley study ('22) in which the milk intake of two pre-school girls (subjects M.O. and K.C.) was increased and decreased at the rate of 250 cc. daily, the percentage utilization values, computed from increments in intake and in retention at intakes ranging from 380 to 950 mg., averaged 20. The percentage retentions, calculated from the same data at the various levels of intake, averaged 29. In Petrunkina's ('34) study on children from 6 to 9 years old, the percentage utilization values (computable for nine of the subjects) ranged from 8 to 50 with a mean of 19%; the percentage retentions averaged 16. These are the only papers found in the literature which permit the computation of percentage utilization. However, considerable data concerning percentage retentions may be obtained from several other studies in which the calcium intakes may not have been in excess of the subjects' requirements. The values, which are inherent in the data of the respective authors, are as follows: 18% for seventeen healthy pre-school children studied by Daniels and co-workers ('34, '35); 23% for Porter-Levin's ('32–33) two healthy children; 20% for six healthy subjects (ranging in age from 5 to 7 years) studied by Bonner et al. ('38); and 22% for nineteen normal and underweight children studied by Wang, Kaucher and Frank ('28). The average for the forty-six subjects who were under 7 years of age is 21%. This figure compares favorably with the percentage retention value reported for the eleven children in this study. Both are considerably less than half the value of 50% which was assumed by Leitch ('37) for the retention of calcium from a 'suitably balanced mixed diet.'

The data secured in this study have a direct bearing upon the fundamental question as to whether or not the maintenance requirement of calcium, as observed in the adult animal, may not be considerably or entirely suppressed in the growing organism. There is considerable evidence in the literature in favor of this possibility. Extremely low excretions of calcium in essentially normal, growing boys subsisting on low-calcium diets were reported by Albright, Bauer, Ropes and Aub ('29) and by Farquharson, Salter, Tibbetts and Aub ('31). Furthermore, Aub, Tibbetts and McLean ('37) observed similar low excretions in a growing boy following a parathyroidectomy. They have suggested that ". . . . a new mechanism had obviously come into play; and both urinary and fecal calcium were being avidly retained in spite of higher blood levels above the so-called kidney threshold. . . ." This 'new mechanism' may be merely a suppression of endogenous losses of calcium brought about by the growing skeleton's intensified demand for calcium and by a simultaneous, inadequate dietary supply. Ellis and Mitchell ('33) showed that growing rats on a severely restricted calcium intake may deposit in their bodies 98% of the calcium consumed, leaving a maximum of only 2% of the intake available for replacement of endogenous losses (assuming a complete alimentary utilization). This efficient utilization of dietary calcium at low levels of intake was confirmed by Fairbanks and Mitchell ('36); they also observed that when a high-calcium diet was fed, variable rates of growth (from 2.38 to 5.08 gm. per day) were associated with constant percentage retentions of dietary calcium. If dietary calcium had replaced endogenous losses, greater percentage retentions of calcium would have been observed during periods of rapid growth and increased food intake, since a greater proportion of the consumed calcium would have been available for growth.

If an appreciable portion of the dietary calcium consumed by the pre-school children of this study had been used to replace endogenous calcium losses (i.e., if an appreciable maintenance requirement of calcium had existed) two effects would have been observed. In the first place, the values for percentage retention of calcium would be less than the values for percentage utilization, since, as was explained before, the latter values are based on intakes which were above the maintenance requirement. Contrary to this expectation, there was no consistent difference between the values for the percentage retention of calcium and those for the percentage utilization. These respective values for each of the eleven subjects were 20 and 18, 14 and 14, 24 and 22, 22 and 25, 20 and 16, 19 and 18, 21 and 21, 19 and 20, 15 and 13, 18 and 19, and 20 and 27, with respective averages of 19.3 and 19.2%. These results are consistent with the hypothesis that the endogenous losses of calcium were entirely suppressed in these children.

The other effect of an appreciable requirement for calcium for maintenance would be an increase in the percentage retention with increasing intake of calcium up to the point of maximum retention. However, in only four of the eleven children was a greater percentage retention of calcium observed on the higher calcium level, and since the averages for the eleven subjects during periods II and III are identical (19.2%), it is safe to suggest again that no appreciable portion of the dietary calcium could have been used in replacing the endogenous calcium lost by way of the intestinal tract and kidneys.

The above reasoning implies that the calcium of the basal foods (which supply approximately 30% of the total calcium during period II) is utilized to an extent no greater than is that of milk calcium, i.e., 20%. If the calcium of these foods were utilized to a greater degree, then a maintenance requirement for calcium must be assumed in order to account for the balance data secured. A utilization of 100% of the basal-food calcium would be consistent with an average daily maintenance requirement (endogenous loss) of 5 mg. per kilogram of body weight. Even if the non-milk calcium had been utilized completely, the 10-mg.-per-kilogram value used by Leitch ('37) in her estimation of children's requirements for dietary calcium would have resulted in negative balances for all six boys during period II and for four boys during period III, and also would have resulted in much lower retentions for the girls than were actually observed. Furthermore, if these children had had a maintenance requirement no greater than that computed by Mitchell ('38) from available information on adults (i.e., 3.1 mg. per kilogram) they would have had to utilize, as an average, 64% of the basal-food calcium. However, there is no evidence in the literature of superior availability of the calcium of ordinary foods over that of milk calcium. On the contrary, experiments have shown that human subjects are able to utilize the calcium of milk just as well as, if not better than, that of other foods. If this is true, then the contention that the children did not have an appreciable maintenance requirement for calcium may be accepted.

Ellis and Mitchell ('33) have portrayed the endogenous metabolism of calcium in the growing animal in the following words:

> Calcium ions liberated in the course of catabolism from functional combinations in the tissues and body fluids are probably just as available for the reconstruction of such combinations as are calcium ions picked up from the intestinal tract. In the young growing animal these 'used' ions as well as the calcium ions coming directly from the alimentary tract may be absorbed and retained by the developing bones and the growing tissues at such a rapid rate that, at low levels of calcium

feeding the threshold of excretion by the kidneys and the intestines is never reached.

If this is a true picture of the situation, the re-utilization of calcium ions liberated in the endogenous catabolism of the tissues may not occur at liberal levels of calcium ingestion, or at least not to the same extent as it would at levels commensurate with the requirements for maximum growth. During periods of generous intake, endogenous losses of calcium would appear and a maintenance requirement for calcium, in the usual sense of the term, would develop.

If subsequent research should prove the above hypothesis and establish its general applicability to growing children, information of considerable practical value should result. One of the possible deductions might be that a value for percentage retention would be indicative of the extent to which the growing child can utilize the calcium of the usual mixed diet, or of how well he can utilize the calcium of a specific food when that food contributes the greater part of the total calcium intake. Thus, the values for percentage retention heretofore reported for those children who were fed submaximal levels of calcium could correctly be interpreted as percentage utilization. Another obvious conclusion would be that, in estimating a child's requirement for calcium, provision need be made only for the calcium requirements of growth.

The limited ability of the pre-school child to utilize milk calcium is in marked contrast with that reported by Ellis and Mitchell ('33) in their studies on rats. They reported retention values as high as 98% for this species. In that study, as in the present one, the experiments were conducted in such a manner as would insure maximum values for utilization in each instance, i.e., the subjects were taken during the period of growth, and the level of calcium at which they were fed was not in excess of their minimum requirements for maximum retention of calcium. The striking disparity in the results obtained indicates a tremendous difference in these two species and provides an excellent illustration of the frequently unappreciated fact that, quantitatively, rats and human subjects do not necessarily react similarly. This finding points to the need for more extensive studies on human subjects before satisfactory dietary standards can be promulgated.

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LITERATURE CITED

ALBRIGHT, F., W. BAUER, M. ROPES AND J. C. AUB 1929 Studies of calcium and phosphorus metabolism. IV. The effect of the parathyroid hormone. J. Clin. Invest., vol. 7, p. 139

AUB, J. C., D. M. TIBBETTS AND R. MCLEAN 1937 The influence of parathyroid hormone, urea, sodium chloride, fat and of intestinal activity upon calcium balance. J. Nutrition, vol. 13, p. 635.

BONNER, P., F. C. HUMMEL, M. F. BATES, J. HORTON, H. A. HUNSCHER AND I. G. MACY 1938 The influence of a daily serving of spinach or its equivalent in oxalic acid upon the mineral utilization of children. J. Pediatrics, vol. 12, p. 188.

DANIELS, A. L., M. K. HUTTON, E. KNOTT, G. EVERSON AND O. WRIGHT 1934 Relation of ingestion of milk to calcium metabolism in children. Am. J. Dis. Child., vol. 47, p. 499.

DANIELS, A. L., M. K. HUTTON, E. M. KNOTT, O. E. WRIGHT AND M FORMAN 1935 Calcium and phosphorus needs of pre-school children. J. Nutrition, vol. 10, p. 373.

ELLIS, M., AND H. H. MITCHELL 1933 The effect of the pasteurization of milk on the utilization of its calcium for growth in the rat. Am. J. Physiol., vol. 104, p. 1.

FAIRBANKS, B. W., AND H. H. MITCHELL 1936 The relation between calcium retention and the store of calcium in the body, with particular reference to the determination of calcium requirements. J. Nutrition, vol. 11, p. 551.

FARQUHARSON, R F., W. T. SALTER, D. M. TIBBETTS AND J. C AUB 1931 Studies of calcium and phosphorus metabolism. XII. The effect of the ingestion of acid-producing substances. J. Clin. Invest., vol. 10, p. 221.

GRANDPREY, M. B. 1933 Range of variability in weight and height of children under six years of age. Child Devel., vol. 4, p. 26.

KRAMER, M. M., E. LATZKE AND M. M. SHAW 1928 A comparison of raw, pasteurized, evaporated and dried milks as sources of calcium and phosphorus for the human subject. J. Biol. Chem., vol. 79, p. 283.

KRAMER, M. M., M. T. POTTER AND I. GILLUM 1931 Utilization by normal adult subjects of the calcium and phosphorus in raw milk and ice cream. J. Nutrition, vol. 4, p. 105.

LEITCH, I. 1937 The determination of the calcium requirements of man. Nutr. Abs. and Rev., vol. 6, p. 553.

MALLON, M. G., L. M. JOHNSON AND C. R. DARBY 1932 A study of the calcium retention on a diet containing American Cheddar cheese. J. Nutrition, vol. 5, p. 121.

MITCHELL, H. H. 1938 The dietary requirement of calcium and its significance. Monograph Series entitled Nutrition, edited by E. F. Terroine.

MITCHELL, H. H., AND F. J. MCCLURE 1937 Mineral nutrition of farm animals. Bull. Nat. Research Council. No. 99, p. 51.

OUTHOUSE, J., G. M. KINSMAN, D. SHELDON, I. J. TWOMEY, J. SMITH AND H. H. MITCHELL 1939 The calcium requirement of five pre-school girls. J. Nutrition, vol. 17, p. 199.

PETRUNKINA, A. 1934 Über die Belanzen von Stickstoff, Kalk, Magnesium, Phosphor und Eisen bei Kindern von 7–8 Jahren. Z. Kinderheilk, Bd. 56, S. 219.

PORTER-LEVIN, T. 1932–1933 Calcium and phosphorus metabolism of normal pre-school children. I. On diets containing plain and irradiated cereals. J. Am. Dietetics Assoc., vol. 8, p. 482.

SHERMAN, H. C., AND E. HAWLEY 1922 Calcium and phosphorus metabolism in childhood. J. Biol. Chem., vol. 53, p. 375.

WANG, C. C., M. KAUCHER AND M. FRANK 1928 Metabolism of undernourished children. IV. Calcium metabolism. Am. J. Dis. Child., vol. 35, p. 856.

WILLARD, A. C., AND K. BLUNT 1927 A comparison of evaporated with pasteurized milk as a source of calcium, phosphorus and nitrogen. J. Biol. Chem., vol. 75, p. 251.

RELATIONSHIP BETWEEN CAROTENE, BLINDNESS DUE TO CONSTRICTION OF THE OPTIC NERVE, PAPILLARY EDEMA AND NYCTALOPIA IN CALVES[1]

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SEVEN FIGURES

(Received for publication January 11, 1939)

In 1935 Moore, Huffman and Duncan ('35) summarized the previous literature and reported on the results of observations of twenty-four cases of blindness of a nutritional type associated with a constriction of the optic nerve. The authors were reluctant to state that this blindness was due to vitamin A deficiency because of the bone changes involved, lack of corneal involvement and because no direct unimpeachable evidence was at hand to support such a statement. It was pointed out, however, that if vitamin A deficiency was responsible for an increased intracranial pressure the bony malformations might then be accounted for. Evidence of increased intracranial pressure is to be reported in this paper and resulted from the suggestion received in a paper reported by De Schweinitz and De Long ('34) who observed blindness and papillary edema in Guernsey calves associated with perivascular edema in the brain tissue. They suggested that the type of blindness was due to inheritance. However, the evidence submitted in this paper will show that a relationship exists between the condition reported by them and by Moore, Huffman and Duncan ('35).

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[1] Journal article 346 (n.s.) Michigan Agricultural Experiment Station. From a portion of a thesis presented to the Graduate School of Michigan State College in partial fulfillment for the Ph.D. degree.

Mellanby ('38) has noted widespread nerve degeneration in young dogs fed diets deficient in vitamin A and rich in cereals. Complete deafness was present in some of the animals which he attributed to nerve degeneration due to an overgrowth of bone of the labyrinthine capsule. He expressed the belief that the degeneration of other nerves might be due to pressure caused by overgrowth of bone in the various foramina.

Guilbert and Hart ('35) established the relationship of night blindness and vitamin A deficiency in the bovine and by use of the night blind condition worked out the minimum requirements for vitamin A. In their paper they expressed the opinion that the blindness reported by Moore et al. ('35) was due to vitamin A deficiency but offered no direct evidence to support the belief. Kuhlman, Gallup and Weaver ('36) reported the development of xerophthalmia and a permanent type of blindness in calves fed a ration of beet pulp and cottonseed meal. Vitamin A supplements cured the xerophthalmia but had no effect on the permanent type of blindness. Phillips and Bohstedt ('38) report the preventive and remedial effects of carotene in oil on rabbits which developed a syndrome of symptoms similar to calves. The rabbits, however, failed to develop a constriction of the optic nerve and no ophthalmoscopic observations were reported.

Hale ('35) published data showing that vitamin A deficiency produced congenital anophthalmos and other abnormalities in swine.

EXPERIMENTAL

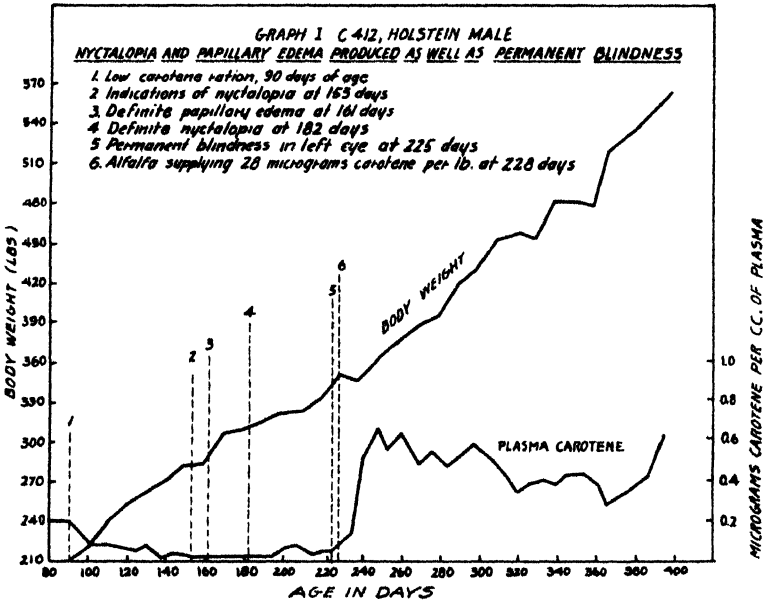
Subsequent to the previous report it was found that cod liver oil in which the vitamin A was destroyed by aeration at an elevated temperature did not prevent the constricted nerve or permanent type of blindness from developing in young calves. Another series of calves was used in which 'caritol' was diluted with cottonseed oil and then homogenized into skim milk so that the fat content of the remade milk was 2%. The carotene content of the milk was so adjusted that the calves received one and two times the minimum requirement suggested by Guilbert and Hart ('35). The calves were started on the milk when 1 week to 10 days old. However, most of the calves died at an early age of pneumonia so that no data were obtained relative to blindness. It was then decided to raise the calves on whole milk and grain to about 90 days of age and then feed skim milk together with a low carotene grain ration consisting of 240 pounds barley, 180 pounds rolled oats, 180 pounds wheat bran, 60 pounds linseed oil meal and 8 pounds salt. This ration contained from 0.5 to 0.7 micrograms of carotene per gram so that the calves received 2 to 3 micrograms per pound of body weight from this source.

After the animals were placed on this ration, ophthalmoscopic examinations were made weekly. The calves were also tested for night blindness each week by attempting to run them into objects and watching their behavior in dim light, a method similar to that used by Guilbert and Hart ('35). Blood plasma carotene determinations were also made each week according to a method developed by the author ('38). Carotene extractions on the hays and feeds used were made according to the modification by Peterson et al. ('37) of the Guilbert method and the carotene determined by the use of a photoelectric colorimeter. The calves were weighed every 10 days at which time adjustments in the amounts of carotene to be fed were made.

The results obtained in three typical cases will be described in detail although observations have been made on many more animals.

Calf C412 was placed on the low carotene ration at 90 days of age, the principal results being shown in graph 1.

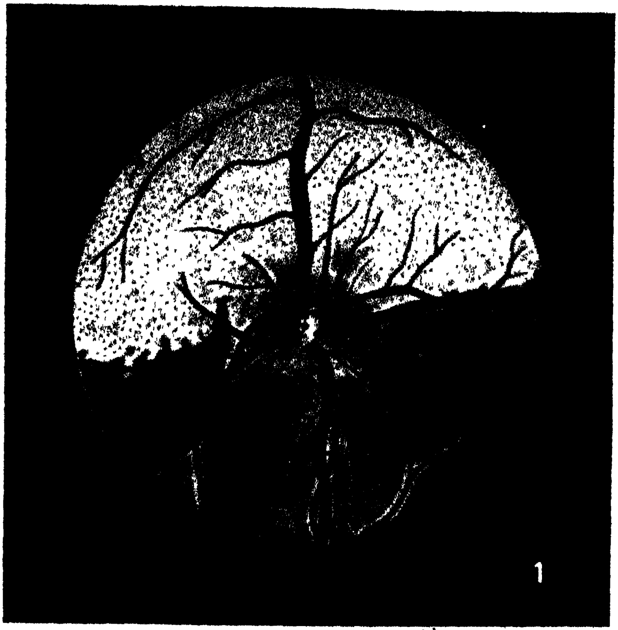
At 106 days of age, a folding of the lens capsule was noted on the posterior pole of the right eye and at this time it was observed that the tapetum lucidum of the retina of both eyes was quite bleached. At 161 days the nerve head of the right eye showed definite swelling and the nerve margins were entirely blurred as shown in figure 2. The nerve head showed a choking of 1½ diopters. At 182 days definite night blindness was present although different degrees of night blindness had been observed after 153 days. Also at 182 days the left eye showed a definite edema with a choking of 0.5 to 1 D of the disc. It was also noted that some of the small vessels of the nerve head were quite dilated. At 203 days, the edema had increased considerably in the left eye so that there was a choking of 2 D. At this time also many of the capillaries and small vessels at the edge of the nerve head showed considerable fusiform dilation. At 225 days the pupil of the left eye did not react to light stimulation and showed mydriasis. By means of covering each eye separately it was found that in daylight the calf was completely blind in the left eye but could still see well out of the right eye. However, in the dark the animal showed complete night blindness in the right eye. The



Graph 1.

Fig. 1 Normal bovine fundus oculi.

Fig. 2 Fundus showing papillary edema and bleaching of the tapetum lucidum in vitamin A deficiency.



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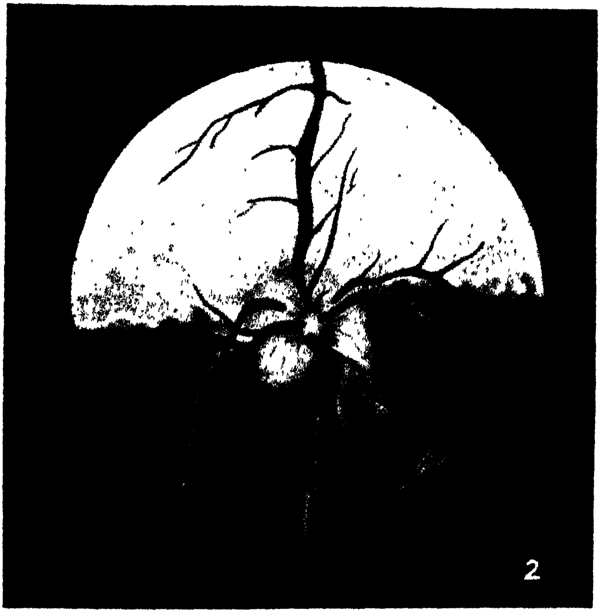
A. Rudolph



2

A. Rudolph

nerve head of the right eye showed edema and some congestion but none of the smaller vessels were dilated. Both eyes showed some exophthalmus.

At 228 days carotene was added in the form of alfalfa hay to the ration at a rate intended to supply 28 micrograms of carotene per pound of body weight in an attempt to preserve the sight of the right eye. The attempt was successful for at the end of 342 days the calf could still see. The sight of the left eye never returned due most likely to a constriction of the optic nerve where it passes through the optic foramen. At 290 days the tapetum lucidum of both eyes showed considerable orange yellow color. The nerve head of the right eye showed a choking of 2 diopters. The nerve head of the left eye looked quite smooth and was quite dark in color indicative of atrophic changes. The vessels of this eye also appeared considerably smaller than normal.

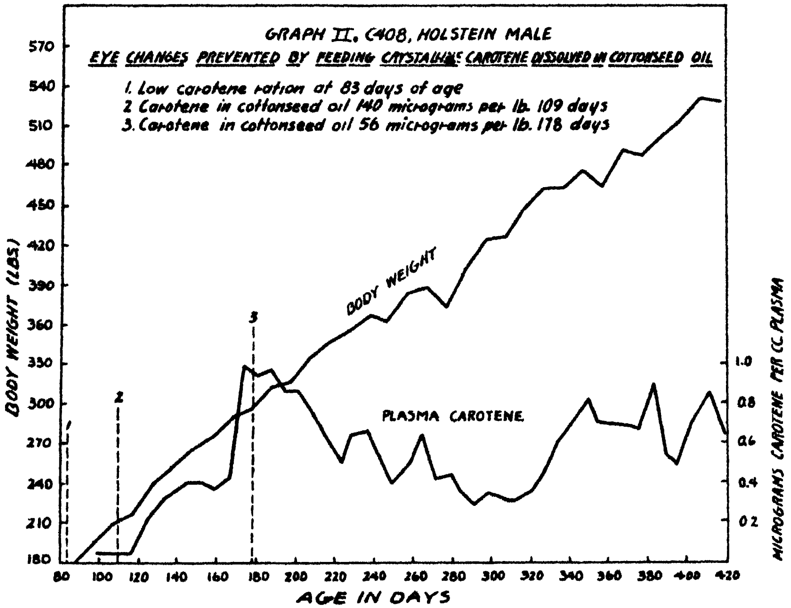
It will be noted from graph 1 that the addition of alfalfa caused an immediate rise in the plasma carotene of this calf.

Calf C408 was placed on the low carotene ration at 83 days of age and at 109 days crystalline carotene dissolved in cottonseed oil was added to the ration at the rate of 140 micrograms per pound. An immediate response in plasma carotene was noted as shown in graph 2. At 178 days the carotene level of the ration was dropped to 56 micrograms and there was an immediate drop in the level of carotene in the plasma. This animal at no time showed any indication of nyctalopia or evidence of papillary edema throughout the period of the experiment. There was some traumatic injury to the cornea and clouding of the anterior chamber as well as some slight hemorrhage on the retina of the right eye.

Calf C411 was placed on the low carotene ration at 90 days of age and at 128 days showed some indications of night blindness but no ophthalmoscopic alterations were observed except bleaching of the retina. Since it was the purpose of the experiment to prevent the development of ocular changes in calves, C408 and C411, the carotene was added immediately to the ration of the latter at the rate of 140 micrograms. At

188 days carotene feeding was discontinued for 10 days and resumed at the 56 microgram level. Throughout the experiment this calf showed no ocular changes.

The carotene[2] added to the ration of the last two calves, C408 and C411, was obtained in crystalline form. One gram was dissolved in 2 liters of cottonseed oil by placing the flask in hot running tap water for about 6 hours with frequent shaking.



Graph 2.

In addition to the observations on calf C412 papillary edema has been noted by the author in eighteen calves and eight mature cows on low vitamin A rations. It has also been observed in three calves at birth whose dams received low vitamin A rations. Nyctalopia has also been seen many times and could be cured in 8 to 10 days by feeding carotene in the form of alfalfa hay. Space does not permit reporting these cases in detail although the results with the larger group have contributed to the ultimate conclusions reached in this paper.

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[2] Purchased from S. M. A. Corporation.

DISCUSSION

The results obtained with calf C412 clearly show that the ration used in this experiment produced nyctaolpia, papillary edema and permanent blindness. The permanent type of blindness which developed in the left eye was prevented in the right eye by the addition of carotene in the form of alfalfa hay. It is quite certain that blindness would have developed in the right eye also had not carotene been administered since in all previous observations blindness has always become bilateral.

The results with calves C408 and C411 give indisputable evidence that crystalline carotene added to the ration prevented the development of nyctalopia, papillary edema and the permanent type of blindness due to constriction of the optic nerve. One must therefore admit that a deficiency of carotene permits these conditions to develop and that the permanent blindness reported by other investigators was probably due to a vitamin A deficiency.

It will be noted that no mention was made regarding the development of xerophthalmia or keratitis. None was observed in these studies. As a matter of fact, in the extensive experiments on vitamin A deficiency in cattle made at this station only a few very mild cases of keratitis have been noted. None have proceeded to the extreme stage of inflammatory reaction of the eye structures even though many animals have died from vitamin A deficiency. The reason for this is not clear. It seems possible that the absence of dust and perhaps certain bacteria in the barns was responsible. Attempts to infect a susceptible calf's eye with the exudate from a rat's eye which showed xerophthalmia were unsuccessful.

The cause of the constriction of the optic nerve is apparently due to bone pressure on the nerve as shown in figure 4 B. That the bony canal is altered in structure and becomes smaller in this condition is also shown in figure 5. As stated previously it is difficult to associate vitamin A deficiency with any bony malformation but if evidence of increased intracranial pressure could be obtained it might explain the cause of the blindness. Such evidence seemed to be present since the nerve heads of these calves showed definite papillary edema as reported by De Schweinitz and De Long ('35) and shown in figures 2 and 3 B. Increased intracranial pressure exerted over a period of time could account for the displaced bone development since the brain itself lies directly above the optic canal. In a young calf the optic canal is very short, less than ¼ inch in length. In a mature animal it is 1½ inches

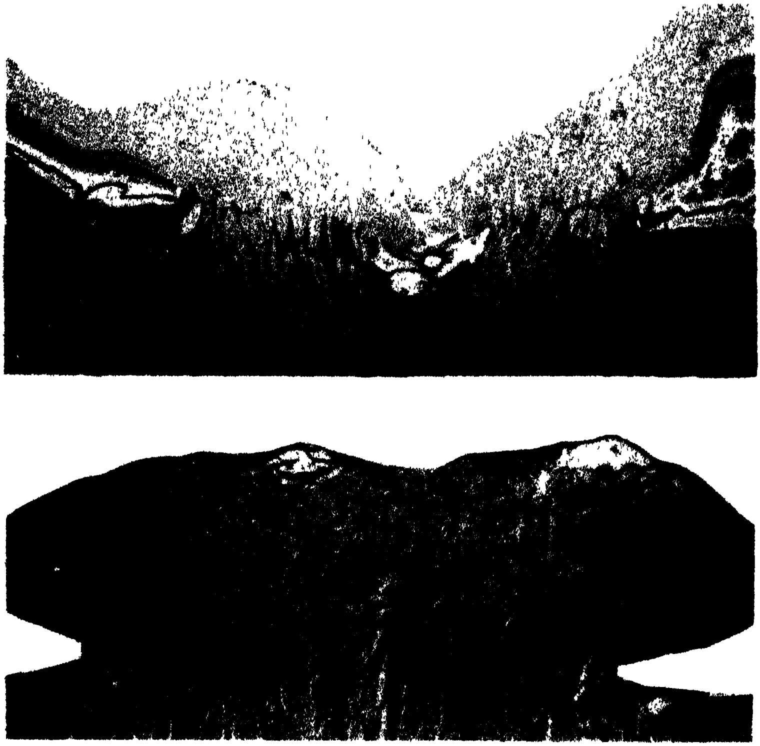

Fig. 3 A. Longitudinal section of a normal optic papilla. × 24. B. Longitudinal section optic papilla showing edema in vitamin A deficiency. × 24.

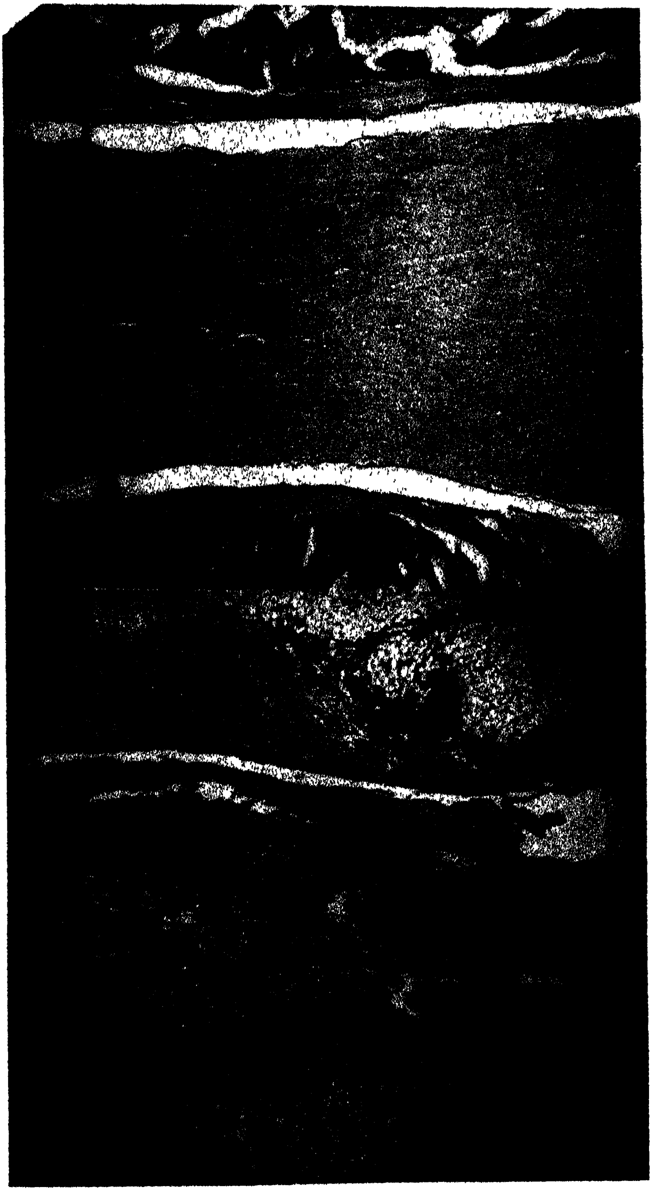

Fig. 4 A. Longitudinal section of a normal optic nerve and bony canal. × 16. B. Longitudinal section showing degeneration of the optic nerve and stenosis of the bony canal. × 16.

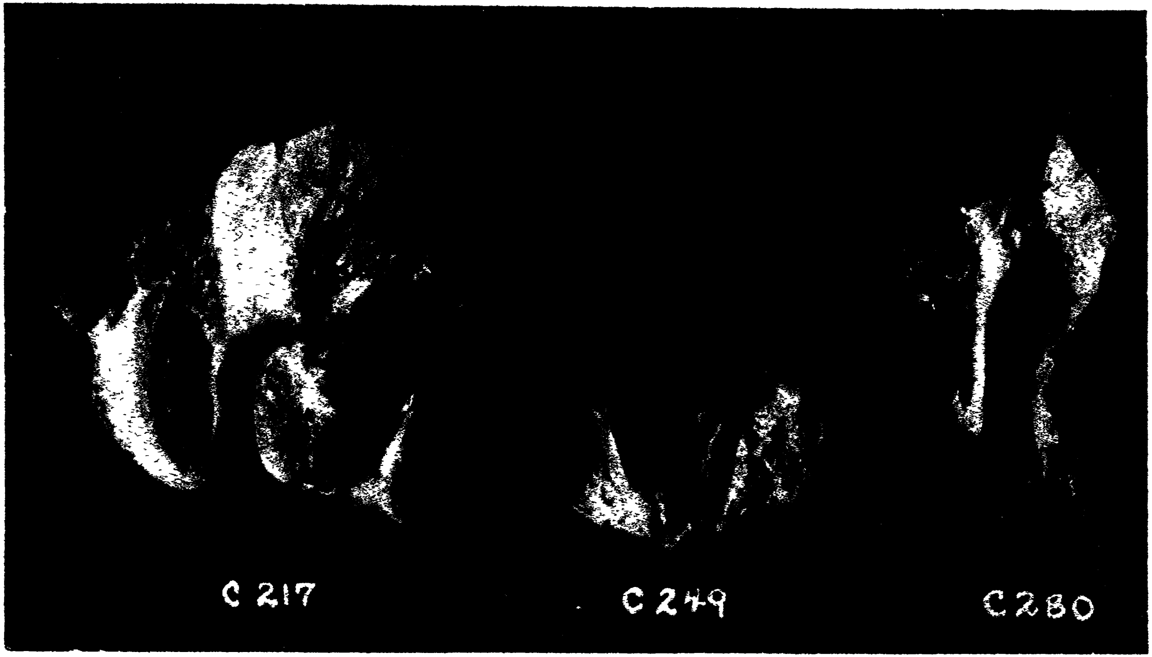

Fig. 5 Showing bony canals of blind and normal calves. C217 and C249 from blind calves showing stenosis; C280, normal.

in length so that over a 2½-year period there is considerable growth. Further evidence of this theory of constriction of the optic nerve is supported by the fact that mature cows develop papillary edema on vitamin A deficient rations but never show the permanent type of blindness. The reason they do not acquire the permanent type of blindness is because the bony canal has fully developed and calcified and therefore no abnormal growth anomaly results. Further evidence of increased intracranial pressure is shown by the condition of syncope, spasms and incoordination all of which are alleviated by vitamin A administration.

De Schweinitz and De Long ('35) did not note constriction of the optic nerve or a stenosis of the optic canal in their cases. In some cases examined by the author these changes were hardly discernible and unless great care is used in dissection they may be missed. It can be argued that very little pressure on the nerve is needed to produce a state of blindness. If the deficiency was corrected at this time the nerve and bony canal would probably show little change. However, if the deficiency was continued a stenosis of the canal and degeneration of the nerve would be produced. That the process can be arrested quite readily was demonstrated in the case of calf C412.

The particular cause of the increased intracranial pressure was not determined although De Schweinitz and De Long ('35) believed it was due to perivascular edema in the brain tissue and showed slides purportedly demonstrating a large perivascular spacing. In the present study it was impossible to secure sections from calves in which the perivascular spacing could not be considered an artifact even though several technics were tried. However, this does not alter the supposition that increased intracranial pressure was present.

Guilbert and Hart ('35) have expressed the belief that the degeneration of the optic nerve was due to vitamin A deficiency per se. However, in the light of present evidence this idea is hardly tenable since the constriction of the nerve always occurs where it passes through the optic canal. Histological evidence of degeneration of nerves other than the optic have not been reported for the bovine in vitamin A deficiency. Furthermore, older cows in which the optic canal has formed do not develop these constrictions. Degeneration of any part of the optic nerve other than at the optic foramen in the calf could easily be accounted for by a decreased blood supply to the part, pressure due to increased intracranial pressure and the pinching effect of the malformed optic canal. Evidence of circulatory disturbance was found in one case in which there was a cystic development in Cloquet's Canal directly anterior to the entrance of nerve into the optic canal. Further evidence of disturbance in the region of the cranial cavity was noted by the quite frequent presence of cysts in the pituitary. The cysts usually occurred between the two lobes and were sometimes larger than the pituitary.

The development of papillary edema associated with vitamin A deficiency has not been reported in other animals. However, one might expect such to occur especially where a condition of syncope and incoordination exists. Incoordination in vitamin A deficiency may not be correlated with myelin degeneration according to work reported by Eveleth and Biester ('37) on pigs. It is entirely possible that the myelin degeneration noted in vitamin A deficiency by various investigators is due directly to increased intracranial pressure and not to vitamin A deficiency per se. Vitamin A may possibly function to regulate the intracranial pressure.

Blindness due to constriction of the optic nerve associated with stenosis of the optic canal has not been reported as occurring in other species and may be peculiar to the bovine because of the particular structure and relationship of cranial cavity and optic canals.

The development of nyctalopia in the calves confirms the observations reported by Guilbert and Hart ('35). Nyctalopia and papillary edema occur at about the same time during the deficiency period but do not necessarily develop simultaneously. The reason for the two conditions not developing at the same time is that they are due to different processes. The papillary edema is probably due to development of intracranial pressure while the nyctalopia is due to depletion of the vitamin A content of the retina in which it is apparently necessary for the regeneration of visual purple. The development of both conditions, however, is due to vitamin A deficiency and they therefore may develop simultaneously also.

It appears that once the papillary edema develops it takes a considerable period for it to recede. In one calf which received carotene at a low level there was little or no recession even after 312 days of supplemental feeding. In another there was a recession of the edema after about 6 months of uninterrupted supplemental feeding. The same slow recession has been noted in mature cows even when they were turned to pasture.

Ophthalmoscopic observations offer a means of diagnosis of vitamin A deficiency in calves if it has proceeded to the point where nyctalopia has developed. Even after the curing of the night blindness ophthalmoscopic observation will inform the observer, by the presence of papillary edema, whether there has been a vitamin A deficiency during the previous 4 to 6 months. In mature cows, other retinal changes take place in vitamin A deficiency which will be reported later.

The amount of vitamin A reserves during the later stages of depletion could to some extent be gauged by the color of the tapetum lucidum of the retina. As depletion progressed the orange yellow color disappeared and was replaced by a pale light blue color as illustrated in figure 2 giving the retina a bleached appearance. The observations on retinal color must be made without exposure of the animals to bright light on the day of examination. It was the usual procedure therefore to leave the calves in the barn the day on which the ophthalmoscopic observations were to be made and to administer the mydriatic a short while before examination. On dark days it was usually unnecessary to administer a mydriatic.

The level of plasma carotene gave considerable information concerning the vitamin A reserves at the low levels at which carotene was fed in these experiments. As a rule the carotene content of the blood plasma remained below about 0.13 micrograms per cubic centimeters for a considerable period of time before the development of nyctalopia and ocular changes. These low values were obtained for calves of the Holstein and Ayrshire breeds but would probably be higher for the Guernsey and Jersey breeds. The response to the administration of carotene was usually immediate except when the amount was small. C408 responded immediately to the administration of the crystalline carotene in cottonseed oil as shown in graph 2.

Studies are now in progress in which the actual cerebral spinal pressure measurements are made by spinal puncture and the use of a manometer. The pathological changes concerned with the increased pressure are also being studied.

SUMMARY AND CONCLUSIONS

1. Calves fed low carotene rations develop nyctalopia, papillary edema and a permanent type of blindness associated with a constriction of the optic nerve.
2. The development of these conditions can be prevented by supplementing the low carotene ration with crystalline carotene dissolved in cottonseed oil. Consequently these conditions appear to be a part of the syndrome of vitamin A deficiency in calves.
3. The development of the constriction of the optic nerve is associated with a stenosis of the optic canal, papillary edema and probably increased intracranial pressure.
4. The development of papillary edema and nyctalopia are separate processes but both are due to vitamin A deficiency.
5. Vitamin A deficiency in calves is usually not associated with the development of keratitis.
6. Vitamin A deficiency in calves is associated with a condition of syncope, most likely produced by increased intracranial pressure.
7. Vitamin A deficiency in calves may be diagnosed by the presence of both papillary edema and nyctalopia.

8. Papillary edema persists for a considerable period after feeding carotene.

9. Under proper conditions of observation bleaching of the tapetum lucidum of the retina is a good guide for the diagnosis of vitamin A deficiency in the bovine.

10. Plasma carotene determinations of the bovine also give an indication of the vitamin A reserve where the colorless vitamin A is not being fed and the carotene intake is somewhat limited.

The author wishes to express his appreciation to Dr. C. A. Hoppert of the chemistry department, Dr. E. T. Hallman of the pathology department, Dr. C. F. Huffman of the dairy department and to Dr. J. O. Wetzel of Lansing for their aid and interest in carrying out this work.

LITERATURE CITED

De Schweinitz, G. E., and Pierce De Long 1934 Blindness and papilledema in Guernsey calves. Arch. Ophthal., vol. 2, p. 194.

Eveleth, D. F., and H. E. Biester 1937 The significance of myelin sheath degeneration and its relation to incoordination. Am. J. Path., vol. 13, p. 257.

Kuhlman, A. H., W. D. Gallup and Earl Weaver 1936 Two types of blindness in cattle and their possible relation to vitamin deficiency. J. Dairy Sci., vol. 19, p. 433.

Guilbert, H. R., and G. H. Hart 1935 Minimum vitamin A requirement with particular reference to cattle. J. Nutrition, vol. 10, p 409.

Hale, Fred 1935 The relation of vitamin A to anophthalmos in pigs. Am. J. Ophthal., vol. 18, p. 1087.

Mellanby, E. 1938 Nerve degeneration and bore hypertrophy induced in young animals by diet. Proc Physiol. Soc., J. Physiol., vol. 93, p. 42.

Moore, L. A., C. F. Huffman and C. W. Duncan 1935 Blindness in cattle associated with a constriction of the optic nerve and probably of nutritional origin. J. Nutrition, vol. 9, p. 533.

Moore, L. A. 1938 The determination of blood plasma carotene in the bovine with a photoelectric colorimeter. In process of publication in the Journal of Dairy Science.

Peterson, W. J., J. S. Hughes and H. F. Freeman 1937 Determination of carotene in forage. Ind. and Eng. Chem. Anal Ed., vol. 9, p. 71.

Phillips, P. H., and G. Bohstedt 1938 Studies on the effects of a bovine blindness producing ration upon rabbits. J. Nutrition, vol. 15, p. 309.

THE INFLUENCE OF DIFFERENT LEVELS OF MILK INTAKE ON THE ACID-BASE MINERAL BALANCES OF CHILDREN

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There is in the literature a dearth of scientific data on the complete acid-base mineral balance of children consuming a general mixed diet (Sawyer, Baumann and Stevens, '18; Cooley et al., '35). Likewise, little is known of the changes that take place in the acid-base mineral balances during childhood in response to any dietary change such as varying the levels of milk incorporated in a known mixed basal dietary. The present paper has been designed to contribute information on these and other factors pertinent to the chemistry of growth of children.

Growth and maintenance depend upon the intake, utilization and excretion of nitrogen and the individual acidic and basic elements. Each of these elements has specific and varied physiologic functions and contributes to the formation of a part of the body. One of the most important functions of the human organism as a whole, and one of the least understood, is the utilization of minerals in a constant attempt toward maintenance of the acid-base equilibrium. The problem is more complex during growth when equilibratory amounts of the acidic and basic mineral elements must be retained for the usual metabolic processes of maintenance, but, in addition to the acidic and basic minerals, a sufficient amount of base-forming minerals termed 'excess base' is required to take care of the growth needs in the enlargement of the body and its organs.

The state of the mineral metabolism of a subject may be expressed as his acid-base mineral balance, given in milliequivalents (meq.) of 'excess base.' The fact that certain elements play primary roles in particular physiologic functions, such as calcium and phosphorus in bone formation, and phosphorus and sulfur in the synthesis of body protein, has led to an emphasis of their individual metabolism in recent years.

It has been stated (Greenwald, '22) that, for man, no relation between metabolism and acid and alkaline diets has been proved; nevertheless Shohl has pointed out (Shohl, '23) that alkaline diets are essential in infancy, where growth is rapid, and are strongly indicated in pregnancy and lactation. Despite the lack of clear-cut evidence of a requirement of excess amounts of acid or base in the food of children, milk is an alkaline food and in the growing child alkali in excess of acid must be stored (White House Conference on Child Health and Protection, '32). Inasmuch as milk yields an alkaline mineral residue containing significantly preponderant amounts of the basic elements (calcium, magnesium, sodium and potassium) over those of the acidic elements (phosphorus, chlorine and sulfur) and does change the percentage and proportions of the various elements in the entire diet, the present study is an attempt to evaluate the effect of different levels of milk intake on the acid-base mineral retentions of growing children over a considerable period of time.

In interpreting the changes in acid-base mineral balances of seven average, healthy children, ages 5 to 10 years, when ingesting different quantities of milk in their diet, it has been possible to consider factors of control and environment—factors of great importance and too often minimized, but adequately controlled during this study due to the facilities made available by the complete cooperation of the Methodist Children's Village, Detroit.[1]

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[1] Details of the control, environment and care and handling of the children, together with the measurements taken and the methods of collecting and sampling the foods and excreta for the 5-day balance periods, are contained in succeeding publications (Hummel, Hunscher and Macy, in press; Souders, Hunscher, Hummel and Macy, in press).

EXPERIMENTAL

The children selected were those whose medical records[2] showed a minimum of illnesses, none of which were extensive or severe, and the absence of known untoward hereditary factors in their histories; who were normal in weight for height by the standards of Meredith ('35) and Boynton ('36), and whose roentgenograms indicated skeletal development and maturity within normal limits.[3] Throughout the study the subjects received diets composed of twenty-one common foods[4] easily obtained throughout the year. The basal foods plus 400 gm. of milk yielded daily an average of 1676 calories (79.3 per kilogram of body weight); approximately 70 gm. of fat (3 per kilogram of body weight); 212 gm. of carbohydrate (9 per kilogram of body weight); 57.35 gm. protein, 5% of the fresh diet, and 17.8% of the dry weight (2.5 gm. per kilogram of body weight); 0.91 gm. calcium (0.041 per kilogram of body weight); 1.25 gm. phosphorus (0.056 per kilogram of body weight). Three young women, trained in nutrition and dietetics, quantitatively prepared and served the food and collected all the metabolic specimens which were analyzed[5] by the staff of the Research Laboratory of the Children's Fund of Michigan.

The milk used in the diets was pasteurized milk obtained from one creamery and subjected to identical processing and handling. During the initial study period, six children received 400 gm. of milk incorporated with their basal dietary daily for periods of 30 to 65 days (total of 225 experimental days); the other child received 600 gm. for 20 days. During the second period of observation, two of the children received 800 gm. of milk daily for 150 to 160 days (total of 310 experimental days) and the remaining five subjects ingested 1000 gm. daily for 20 to 40 days (total of 140 experimental days). One child was returned to an intake of 400 gm. daily for 20 days after receiving the 1000 gm. feeding daily. The protein, fat and acid-base values of a representative sample of the basal foods, and of 100 gm. of milk, are:

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[2] Marsh W. Poole, M.D., and Charles A. Wilson, M.D., attending physicians at the Methodist Children's Village, kindly made the medical examinations, the physical measurements and were responsible for maintaining the health of the children.

[3] The late T. Wingate Todd, M.D., and C. C. Francis, M.D., Western Reserve University, cooperated generously in a most helpful way by evaluating the skeletal maturity of all the children contacted, particularly those finally chosen for the metabolic study.

[4] The basal diet was composed of: apple, banana, beef, white and whole wheat bread, butter, cabbage, carrots, cheese, eggs, graham crackers, lettuce, milk, peanut butter, potato, shredded wheat or cornflakes, tomato and orange juice, sugar and salt.

[5] The chemical methods, omitted by space requirements, may be obtained from the authors. Methods of analysis will be presented in detail in a forthcoming monograph upon the chemical growth of children.

	Protein *gm*	*Calcium* *meq.*	*Magnesium* *meq.*	*Sodium* *meq.*	*Potassium* *meq*	*Total base* *meq*
Basal foods	46.8	12.4	16.6	73.2	42.5	144 7
100 gm. milk	3.1	5.9	1.0	2.1	3.8	12.8

	Fat	*Phosphorus*	*Chlorine*	*Sulfur*	*Total acid*	*Base minus acid* [1]
Basal foods	45.1	37.1	73.3	31.5	141.9	2.8
100 gm. milk	3.1	5.2	3.0	1.7	9.9	2.9

[1] The acid base mineral value: milliequivalents of 'excess base.'

In the calculation of the acid-base value of food the fact that the organic forms of phosphorus and sulfur do not bind base is a source of error that is recognized but cannot be controlled. During growth, however, it is mitigated somewhat by the fact that part of the phosphorus and most of the sulfur are deposited in organic form in the soft tissues of the body. When the organically bound phosphorus and sulfur are metabolized they contribute acid to the excreta which is not represented as such in the food. Inasmuch as nothing is known of the proportion of the different forms of the several elements in the food it seemed best for all practical purposes, especially in this study of growing children, to consider all of the minerals as entering into the acid-base value and to use 2 as the valence of sulfur and 1.8 as the valence of phosphorus (Shohl, '23). This procedure is justified further on the basis of previous studies (Hummel, Hunscher and Macy, in press; Souders, Hunscher, Hummel and Macy, in press).

The basal diet used in the study, exclusive of milk, contained only 2.8 milliequivalents (28 cc. 0.1 N alkali) of base in excess of acid. The addition of milk to this diet enhanced its basic qualities and thereby caused considerable change in the relative proportions of the various elements and the extent of their contribution to the acid-base value of the food. The quantities of the different elements in the basal foods and the amounts of the same elements which are added by each increase of 100 gm. of milk, have been given in order to bring out more clearly the changes in the proportion of each constituent resulting from dietary change.

The largest difference between the acid-base values of the diets with and without 400 gm. of milk is attributable, for the most part, to the change in calcium content. There was an increase of nearly 200% in this element due to the milk, i.e., 400 gm. of milk contributed almost twice as much calcium to the intake as did all the basal food constituents; or, on the basis of the different levels of milk, each 100 gm. of milk supplied in the diet added 50% as much calcium as was supplied in all the other foods used in the diet. The inclusion of 400 gm. of milk raises the average daily excess base value of the diet from 2.8 to 14.4 milliequivalents, an increase of 11.6 milliequivalents; with 1000 gm. of milk the diet contained more than eleven times as great an excess of base over acid as the foods without any milk.

The next largest contribution of milk to the diet was phosphorus. The 400 gm. portion of milk contained slightly more than 50% of the amount of phosphorus found in all the other foods. When supplied in amounts of 800 gm. or larger it furnished the greater part of the phosphorus in the diet. In addition to calcium and phosphorus, the potassium and chlorine content of the diet was enhanced to a great degree, amounting to 89 and 42% increases, respectively, with the addition of 1000 gm. of milk. Milk is usually considered a poor source of sulfur, yet the sulfur content of the diet is augmented by 21, 31.7, 42.5 and 53% by the addition of 400, 600, 800 and 1000 gm. of milk, respectively. In a like manner the protein in the total diet was increased daily from 59.1 to 77.5 gm. (26.3 to 65.8%), and the fat from 57.5 to 76.1 gm. (27.5 to 68.8%) when the milk levels were changed from 400 gm. to 1000 gm.

RESULTS

The average daily nitrogen and acid-base balances, together with the recumbent length and weight gains of the children, are shown in table 1. All of the subjects stored nitrogen, acid and base during the study. When the food intake was augmented by adding milk the retentions increased, as did the levels of excretion in the acid and base in both the urine and feces. The children with the highest intakes per kilogram of body weight, however, did not always have the highest retentions. D.P., the child with the lowest average daily intake per kilogram of body weight of both acid and base, 7.4 and 8.1 milliequivalents, respectively, showed the lowest average retention of base, 0.7 milliequivalent per kilogram daily, but not the lowest retention of acid. With 400 gm. of milk in the diet, the average excess of base over acid in the intakes was 0.6 milliequivalent per kilogram per day (6 cc. 0.1 N alkali) and the range, 0.5 to 0.7 meq./kg. When the level of milk ingestion was raised to 1000 gm. the average excess of base over acid in the intakes increased to 1.6 meq./kg./day. Four of the seven children showed a storage of excess base on all the levels of milk throughout the study. From the means and ranges of the intakes and retentions of total base, total acid, and excess base a marked correlation was observed.

Although the urine constitutes the major outlet of both acid and base, the urine of these subjects at all times contained an excess of acid over the base and the feces always contained more base than acid. Milk contributed appreciable quantities of fat to the diet, but the excretion of an excess of acid in the urine was practically unaffected by the dietary changes, while the amount of excess base in the feces (probably to a great extent in the form of calcium soaps) increased during ingestion of the larger quantities of milk (unpublished data). These

TABLE 1

Nitrogen and acid-base mineral balances of seven children during periods of ingesting diets differing in milk quantity

CHILD AND AGE	MILK INTAKE	LENGTH OF STUDY	RECUMBENT LENGTH		WEIGHT		NITROGEN	TOTAL BASE				TOTAL ACID				TOTAL BASE MINUS TOTAL ACID			
			Initial	*Daily gain*	*Initial*	*Daily gain*	*Retention*	*Intake*	*Outgo Urine*	*Outgo Feces*	*Retention*	*Intake*	*Outgo Urine*	*Outgo Feces*	*Retention*	*Intake*	*Outgo Urine*	*Outgo Feces*	*Retention*
months	*(gm.)*	*(days)*	*(cm)*		*(kg)*		*(mg/kg/day)*	*(meq per kg per day)*				*(meq. per kg. per day)*				*(meq. per kg. per day)*			
D.P.:																			
99	400	65	135.4	0.034	27.79	0.002	2.1	8.1	5.5	1.9	0.7	7.4	5.9	1.0	0.5	0.7	—0.4	0.9	0.2
101	800	160	137.6	0.008	27.89	0.008	13.6	9.9	6.3	2.4	1.2	8.8	6.9	1.1	0.8	1.1	—0.6	1.3	0.4
W.P.:																			
96	400	30	119.4	0.003	22.88	0.010	7.8	9.5	6.2	2.5	0.8	8.8	7.2	1.2	0.4	0.7	—1.0	1.3	0.4
97	800	150	119.5	0.015	23.19	0.012	35.2	11.3	6.9	3.4	1.0	10.1	7.9	1.5	0.7	1.2	—1.0	1.9	0.3
C.D.:																			
123	600	20	134.2	0.015	33.95	0.028	43.8	9.7	5.0	2.5	2.2	9.2	5.8	1.2	2.2	0.5	—0.8	1.3	0.0
124	1000	20	134.5	0.020	34.50	0.030	60.7	12.4	5.4	3.1	3.9	11.1	6.2	1.4	3.5	1.3	—0.8	1.7	0.4
R.C.:																			
68	400	30	111.8	0.040	19.65	0.014	40.4	11.8	6.9	2.2	2.7	11.2	7.5	1.6	2.1	0.6	—0.6	0.6	0.6
70	1000	35	113.2	0.037	20.13	0.013	54.0	16.3	7.9	3.6	4.8	14.6	9.3	2.1	3.2	1.7	—1.4	1.5	1.6
J.L.:[1]																			
106	400	30	128.3	0.023	25.27	0.008	40.4	9.3	5.3	2.3	1.7	8.8	5.5	1.2	2.1	0.5	—0.2	1.1	—0.4
107	1000	25	129.0	0.025	25.50	0.006	60.2	13.0	6.3	3.3	3.4	11.6	6.9	1.8	2.9	1.4	—0.6	1.5	0.5
P.S.:																			
66	400	30	114.8	0.053	22.30	0.014	18.6	10.4	5.1	2.8	2.5	9.9	6.4	1.5	2.0	0.5	—1.3	1.3	0.5
67	1000	40	116.4	0.068	22.72	0.008	46.0	14.5	6.6	4.2	3.7	12.9	7.9	2.3	2.7	1.6	—1.3	1.9	1.0
C.L.:[1]																			
65	400	40	107.0	0.015	17.85	0.011	28.1	13.0	7.1	3.6	2.3	12.3	8.0	2.0	2.3	0.7	—0.9	1.6	0.0
66	1000	20	107.6	0.050	18.30	0.045	53.5	17.6	9.0	5.0	3.6	15.7	9.8	2.8	3.1	1.9	—0.8	2.2	0.5
67	400	20	108.6	0.080	19.20	0.000	35.2	12.2	6.6	3.0	2.6	11.6	7.5	1.9	2.2	0.6	—0.9	1.1	0.4
Means for 400 gm. intake (245 days)							21.1	10.3	6.0	2.6	1.7	9.7	6.7	1.5	1.5	0.6	—0.7	1.1	0.2
Means for 1000 gm. intake (140 days)							53.7	14.8	7.1	3.8	3.9	13.2	8.1	2.1	3.0	1.6	—1.0	1.7	0.9

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[1] Female.

findings point to the importance of the bowel as a significant excretory pathway in the control of acid-base mineral balance, in spite of the effective mechanism used by the kidney in maintaining acid-base equilibrium of the body.

Table 1 illustrates differences among individuals as well as the changes in mean retentions corresponding to increases or decreases in intake. Considering both increase in intake and individual differences the Analysis of Variance Method (Snedecor, '34) enables us to conclude that the differences in mean retentions of total base, total acid and excess base are all statistically significant for the change from 400 to 1000 gm. of milk; when the change in level of milk intake was from 400 to 800 gm. per day the differences in the mean retentions of total base and total acid were statistically significant. Although the changes in quantity of mineral elements ingested produced corresponding and significant differences in the retentions, it is apparent that the retentions of the different children are primarily characteristic of their inherent physiologic needs.

The children showed gains in recumbent length and weight during the course of the study, but the amounts of these gains seem to have no direct relationship with the quantity of excess base stored. The children all retained nitrogen throughout the study, ranging from 2.1 to 40.4 mg. per kilogram of body weight daily on the 400 gm. level of milk intake to 46.0 to 60.7 mg. during the 1000 gm. intake. The lack of relationship between the amount of storage of excess base and gains in weight or recumbent length is best illustrated by C.L. This child received 400 gm. of milk in her diet for a period of 40 days, during which time she maintained acid-base mineral equilibrium and gained in recumbent length and weight, 0.015 cm. and 0.011 kg. per day respectively. During this period she stored, on the average, 14.9 mg. calcium and 28.1 mg. of nitrogen per kilogram of body weight. For the next 20 days 1000 gm. of milk were ingested daily; the nitrogen and calcium storages rose to 53.5 and 28.9 mg. per kilogram of body weight respectively, and the retention of excess base increased to

0.5 milliequivalent while the gain in recumbent length rose to 0.05 cm. and the weight to 0.045 kg. daily. During the last 20 days of the experiment this child was returned to a 400 gm. level of milk. The storage of excess base dropped only slightly to 0.4 milliequivalent, the nitrogen to 35.2 mg. and the calcium to 15.2 mg. per kilogram daily. The recumbent length rate of gain, however, continued to increase to 0.08 cm. daily, while the weight remained stationary.

These results indicate that neither the recumbent length, the weight gains, nor the excess base balance, alone, give an index to the type of chemical growth that is taking place. The retention of nitrogen is a more exact measure of growth than is the increase in weight, which may be due in a varying degree to body water or fat. Space does not permit discussion of the interrelation of the various acid-base mineral elements in growth, but this will be the subject of future publications. Because of the accuracy and frequency with which the recumbent length measurements and weights were taken, however, they contribute significant information which correlates with the metabolic balances of eleven different chemical elements and constituents in specific types of chemical interrelationships. With the changes to higher levels of milk intake the increased calcium: phosphorus ratios indicate gains in rate of synthesis of skeletal tissue over the rate of soft tissue construction, and bear out the fact that the kind of chemical growth accruing at any particular time is characteristic of the individual child with his peculiar inherent physiologic needs in response to the growth impulse as well as to the type and quantity of food that he consumes.

It seems apparent from these data that the extra base supplied by the additional quantities of milk caused a greater positive base balance in these seven growing children, although unrelated quantitatively to the actual gains as measured by recumbent length and weight. The children were at all times storing both basic and acidic minerals, even on the mixed diet with 400 gm. of milk, but the base balance was not always positive during the lower level of milk ingestion. It was not until 1000 gm. of milk were given daily that the level of excess base retention for these children approximated the 10 cc. of 0.1 N base suggested by Shohl ('23) as being desirable for growth.

SUMMARY

The nitrogen, acid (phosphorus, chlorine, sulfur), base (calcium, magnesium, sodium, potassium) and 'excess base' balances are given for seven healthy children, ages 5 to 10 years, over 715 experimental days, when they were consuming 400, 600, 800 and 1000 gm. of milk daily. Not only are the various quantities of the elements changed in the diet but the proportion of one to the other is appreciably changed when different levels of milk are incorporated in a known fixed dietary.

Due to the inherent physiologic conditions characteristic of the individual child and not always amenable to control or adjustment, each child served as his own control and was observed on two different levels of milk. Again, due to the variability of metabolism from day to day, especially in children, each observation period extended over 20 or more consecutive days on a given intake of milk, but in one case it extended as long as 160 consecutive days. The basal diet was kept constant in quality of mixed foods though the quantity was adjusted to meet the physiologic needs of the individual child; the average daily intake including 400 gm. of milk per kilogram of body weight amounted to: 79.3 calories, 3 gm. of fat, 9 gm. of carbohydrate, 2.5 gm. of protein, 41 mg. of calcium and 56 mg. of phosphorus.

All children were storing both acid and base throughout the entire study but the quantity stored was greater during ingestion of the larger amounts of milk. The mean daily balance of 'excess base' for all the children on the 400 gm. intake of milk was 0.2 meq. (2 cc. 0.1 N base) per kilogram of body weight as contrasted with 0.9 meq. (9 cc. 0.1 N base) during the daily ingestion of 1000 gm. of milk.

LITERATURE CITED

BOYNTON, B. 1936 The physical growth of girls. A study of the rhythm of physical growth from anthropometric measurements on girls between birth and eighteen years. Univ. of Iowa Studies, Studies in Child Welfare, vol. 12, no. 4.

COOLEY, T. B., G. C. PENBERTHY, L. ARMSTRONG, H. A. HUNSCHER, F. COPE AND I. G. MACY 1935 Mineral metabolism. A case of osteopsathyrosis and one of ununited fracture. Am. J. Dis. Child., vol. 50, p. 431.

GREENWALD, I. 1922 A normal diet. Endocrinology and Metabolism, ed. by L. F. Barker, New York and London, vol. 3, p. 413.

HUMMEL, F. C., H. A. HUNSCHER AND I. G. MACY 1939 The influence of fluid irradiated and evaporated irradiated milks on the storage of nitrogen and acid-base minerals in children. Am. J. Dis. Child. (In press.)

MEREDITH, H. V. 1935 The rhythm of physical growth. A study of eighteen anthropometric measurements on Iowa City white males ranging in age between birth and eighteen years. Univ. of Iowa Studies, Studies in Child Welfare, vol. 11, no. 3.

SAWYER, L., L. BAUMANN AND F. STEVENS 1918 Studies of acid production. II. The mineral loss during acidosis. J. Biol Chem., vol. 33, p. 103.

SHOHL, A. T. 1923 Mineral metabolism in relation to acid-base equilibrium. Physiol. Rev., vol. 3, pp. 509.

SNEDECOR, G. W. 1934 Calculation and interpretation of analysis of variance and covariance. Collegiate Press, Ames, Iowa.

SOUDERS, H., H. A. HUNSCHER, F. C. HUMMEL AND I. G. MACY 1939 A comparison of the influence of fluid and evaporated milks on mineral and nitrogen metabolism of growing children. Am. J. Dis. Child. (In press.)

STEARNS, G. 1931 The significance of the retention ratio of calcium: phosphorus in infants and children. Am. J. Dis. Child., vol. 42, p. 749.

WHITE HOUSE CONFERENCE ON CHILD HEALTH AND PROTECTION 1932 Growth and development of the child. Part III. Nutrition. The Century Co., New York and London, p. 213.

THE INFLUENCE OF EXERCISE ON THE GROWING RAT IN THE PRESENCE AND ABSENCE OF VITAMIN A[1,2]

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FIVE FIGURES

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Research literature in the field of nutrition abounds in unexplained variations and discrepancies owing to numerous biological variables which are difficult if not impossible to control. This is especially true of those data obtained in investigations dealing with various phases and forms of malnutrition. Investigators have long recognized certain influencing factors and have attempted to determine the relative magnitude of their respective influences on the ultimate experimental results. The importance of heredity and previous nutritional history of the experimental subjects, together with such factors as age, sex and size, have been rather extensively investigated. Other factors including radiant energy and environmental temperatures and humidities have also received consideration. Although it has been frequently assumed that a relationship exists between the physical activity of an experimental animal and its nutritional response, little actual experimental work has been done toward establishing the relative significance of this factor, and these efforts have been directed mainly from the standpoint of energy requirements.

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[1] Authorized for publication December 9, 1938, as paper no. 876 in the journal series of the Pennsylvania Agricultural Experiment Station.

[2] Presented before the division of biological chemistry at the Milwaukee meeting of the American Chemical Society, September 5 to 9, 1938.

It appears that adequate investigations have not been conducted regarding either the relationship of physical activity to the vitamin requirement of various species of animals or to the effect of vitamin intake on the inclination of such animals to exercise. Yoshiue ('22) reported that vitamin B deficient rats which were exercised died before developing the muscular paralysis typical of this deficiency. Blacklock ('24), on the other hand, has expressed a belief that lack of exercise accelerates and accentuates the ill effects of diets deficient in vitamin B. This author cites the fact that the disease beri beri most frequently occurs in institutions and in those situations where a fixed diet and some degree of confinement are usually maintained. Keith and Mitchell ('23) placed rats receiving a vitamin A deficient diet in cylindrical boxes and rotated the boxes at the rate of thirteen revolutions per minute for periods ranging from 30 to 40 hours per week. As a result of the studies these authors concluded that exercise distinctly hastened the appearance of the symptoms of vitamin A deficiency and hastened the death of young animals receiving vitamin A deficient diets. Similar studies carried out by these authors while employing diets deficient in vitamin B did not show that the requirement for this dietary factor was affected by exercise. In addition to these citations, numerous authors have inferred that they recognized certain influences of exercise on vitamin requirement as well as increased physical activity as the result of adequate vitamin intake, although no data were given in support of such statements.

It would seem that if exercise does influence vitamin requirement some of the variations in individual response of experimental animals comprising a carefully selected group might be due to differences in physical activity of different animals in the group, and also that differences in responses obtained by different investigators might be at least partially explained on the basis of the difference in the degree of freedom allowed the experimental subjects. With these thoughts in mind it appeared that the influence of exercise or activity of the experimental animal on its vitamin requirements and also the effect of vitamin intake on physical activity merited further study. The present report is concerned primarily with the influence of exercise on the development and well being of the growing rat in the presence and absence of vitamin A. Similar studies are now under way with reference to other vitamins.

EXPERIMENTAL

The investigation consisted essentially of a comparison of the physiological activity and growth responses of comparable groups of young rats while being maintained under identical conditions with the exception of the amount of exercise permitted by the types of caging employed and of the vitamin A intake. The experimental equipment consisted of three sets of individual all-metal cages, of which two sets were of rotating type, having a wheel approximately 14 inches in diameter that was equipped with a registering cyclometer. One set of the rotating cages was so arranged on one side of a large table that the wheels could be rotated at a definite speed by means of a continuous belt driven by an electric motor through a gear reducing mechanism. The second set of rotating cages was arranged on the opposite side of the table and the wheel of each cage was adjusted so as to allow uniform freedom of rotation. The third set of cages consisted of our regular laboratory cages, each of which had approximately 120 square inches of floor space. All cages were provided with raised screen floors. Clean paper was kept under the cages.

Healthy rats, 21 to 23 days of age and weighing approximately 45 gm., were used as the experimental subjects. A special effort was made to place comparable animals of the same litter, sex and body weight in the three respective groups. All animals were fed ad libitum a vitamin A deficient basal diet which consisted of: purified casein 18, salt mixture 3, Cell-U-flour 2, dextrinized cornstarch 77 and irradiated yeast 8. Clean distilled water was available at all times.

Each set of animals was subdivided into two groups. Animals of one group received only the vitamin A deficient diet until depleted of their body reserve of this vitamin, while the animals of the second group served as positive controls and in consequence each received 2 U.S.P. units of vitamin A daily. After being depleted, the animals of the former group also received this quantity of vitamin A daily for a period of 35 days.

The animals of the forced exercise group were allowed access to the rotating wheel only during the period of rotation. During the remainder of the time they were confined in the small side cage (60 square inches of floor space) containing the food and water. The animals of the voluntary exercise group and those housed in the stationary cages were allowed free access to the entire cage at all times.

Observations made on a group of normal young rats which received our breeding colony diet, housed in the above type of rotating cage and allowed freedom of exercise, had shown that an average of 1000 revolutions per day was made during a period of 70 days. This number of revolutions was taken, therefore, as the basis for the maximum daily rotation of the forced exercised groups. Previous observations had also indicated that a speed of approximately six revolutions per minute, with this particular type of cage, was the most effective in keeping the animals in constant motion. Speeds less than six revolutions per minute allowed the animals to stop frequently, while greater speeds tended to cause the animals to cling to the wheel and 'ride' instead of exercising as was desired. At the above rate of rotation, approximately 3 hours per day were required to rotate the cages of the forced exercise group 1000 revolutions or a distance of approximately 0.7 mile. This period of rotation was always started in the early forenoon following the weighing of the animals. At the end of the rotation period each animal was again returned to the small side cage containing the food and water where it remained until the following day.

A daily record was made of the weight of the animal, the amount of food consumed, the number of fecal pellets and the number of revolutions made by the wheels of the rotating cages during the previous 24 hours. All fecal matter was removed from the cage pans daily and stored in glass containers. At the end of each week the fecal collection from each rat was dried and weighed. Daily observations were also made of the animals in an effort to detect the onset as well as the relative stage of the symptoms characteristic of vitamin A deficiency. As the body weight of each animal ceased to increase and symptoms of xerophthalmia appeared in one or both eyes, the animal was given daily doses of 2 U.S.P. units of vitamin A as a supplement to the deficient diet (see above). The duration of the experimental periods for all animals ranged from 64 to 71 days, depending upon the time required to deplete the respective animals. Those animals receiving the vitamin A supplement from the beginning of the experiment were continued under observation for a period of 70 days.

DATA

In order to conserve space the data obtained for individual animals have been reduced to group averages and are presented in the following charts (see figs. 1 to 5 inclusive). Only data from those animals which survived the entire experimental period have been used in tabulating these summaries.

DISCUSSION

It was observed at the beginning of the experiment that those animals housed in the rotating cages and composing the forced exercise group were unable to undergo the full 1000 revolutions before becoming completely exhausted. In order to avoid any possible vitiating effect of over exhaustion on subsequent responses, these animals were exercised during the first few days of the experiment to the point of partial exhaustion and the number of revolutions was increased daily until the end of the first week at which time the cages were undergoing the desired 1000 revolutions daily.

It may be observed from the data presented in figure 1 that exercise, under the conditions of this experiment, had a measurable depressing effect on the rate of growth of young rats irrespective of the vitamin A intake. The effect of this factor on the rate of growth was, however, somewhat greater among those animals which had been previously depleted of their body store of vitamin A. These findings are further emphasized by the data presented in figure 2 relative to the effect of exercise on the total change in body weight during the entire experimental period. Here again the animals of

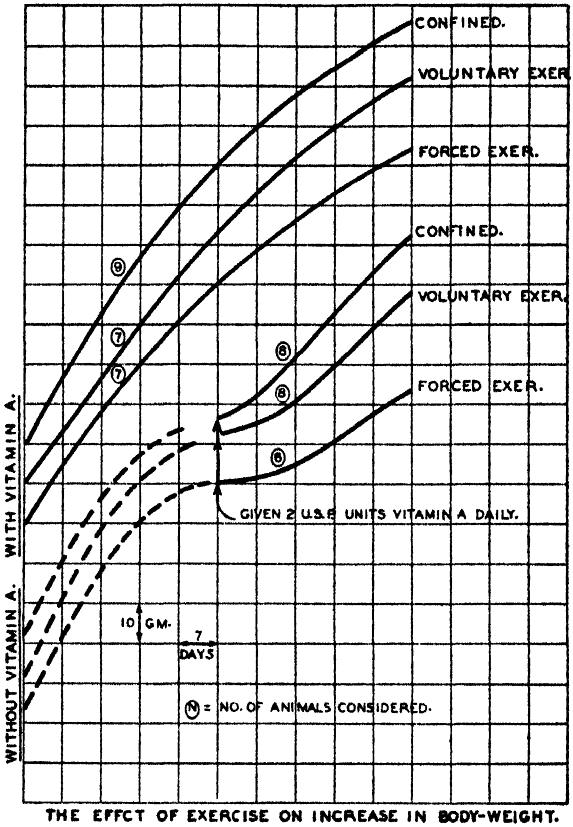

Fig. 1 The effect of different amounts of exercise on the growth rate of young rats while being maintained under comparable dietary conditions except as to their vitamin A intake.

the confined groups, both with and without vitamin A, made the greatest increases in body weight. No attempt was made to determine the chemical nature of the weight increases of the respective groups of animals.

The data presented in figure 2 concerning the total food intake of the various groups of animals are somewhat surprising in that it was expected that greater physical activity would result in greater food consumption in order to compensate for the greater energy exchange involved. Such is not indicated by the data obtained, for in each instance the group of animals undergoing the least amount of exercise consumed the greatest amount of food. Similar results have been reported by Keith and Mitchell ('23). While no explanation can be offered at this time for the suppressing effect of exercise on food intake, it seems highly probable that the physical effects of the 3 hours of rotation during the forenoon are important considerations.

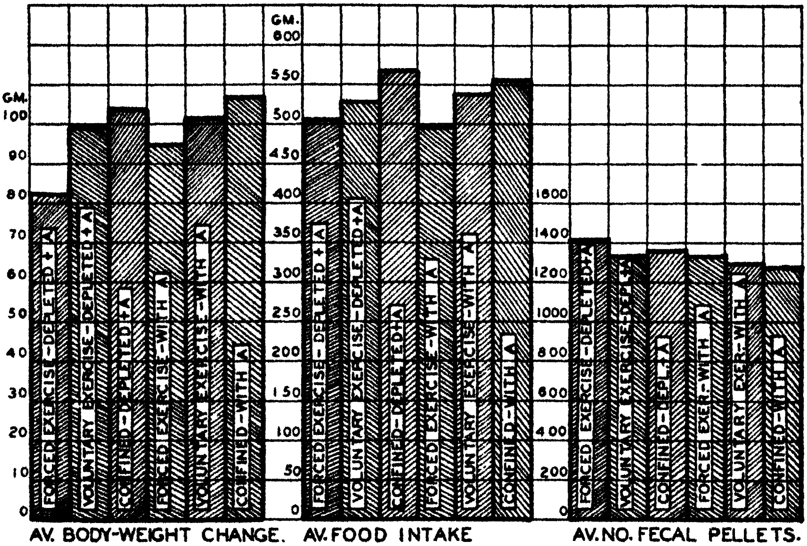

Fig. 2 The relative effect of exercise on the average body weight increase, average food intake and average number of fecal particles voided by young rats receiving a diet with and without vitamin A.

Another unexpected observation is the close similarity in the total amounts of food consumed by the animals of the corresponding groups irrespective of their vitamin A intake. These findings can at least be partially explained on the basis of the high food intake of the depleted animals during the 35 days the vitamin A supplement was fed.

Figure 2 also contains data concerning the effect of exercise on the average number of fecal pellets voided by the different groups of animals during the entire experimental period. It will be observed that while the number of fecal pellets voided by the different groups of rats did not vary widely, in each instance those animals composing the forced exercised groups voided the greatest number of pellets. These findings may be interpreted as signifying a favorable effect of exercise on intestinal motility and, in consequence, on elimination. Exercise is generally believed to have a similar effect on humans.

When one considers the total weight of the fecal matter, the relative proportions of food excreted as fecal matter and the average weight of the fecal pellets voided by the various groups of animals (fig. 3) differences between the groups become more significant. In all instances those animals of the confined groups voided the greatest weight of fecal matter, the greatest percentage of fecal matter and the heaviest fecal pellets. The lower percentage of fecal matter from the animals of the exercised groups might be explained on the basis that these animals were more efficient in the utilization of part or all of the constituents of their food than were comparable groups of animals which were not exercised. The smaller fecal pellets from the exercised animals further indicate increased intestinal motility among the animals of these groups.

From the data presented in figure 4 it may be observed that exercise has a significant effect on the time required for the cessation of growth and for the onset of xerophthalmia among rats receiving a diet deficient in vitamin A. The values given in these graphs represent the averages for all animals of the respective groups. It may be seen that it required, on

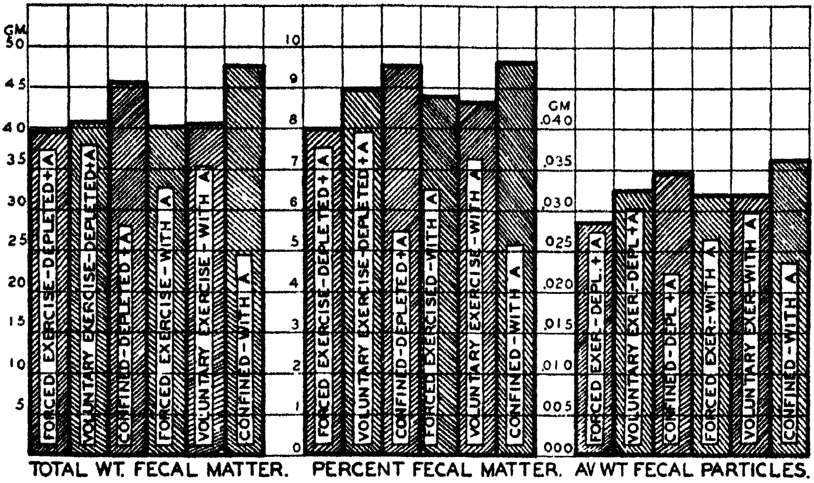

Fig. 3 The effect of exercise on the total weight of fecal matter, percentage of fecal matter and average weight of fecal particles voided by young rats while receiving diets comparable in all respects except as to the vitamin A content.

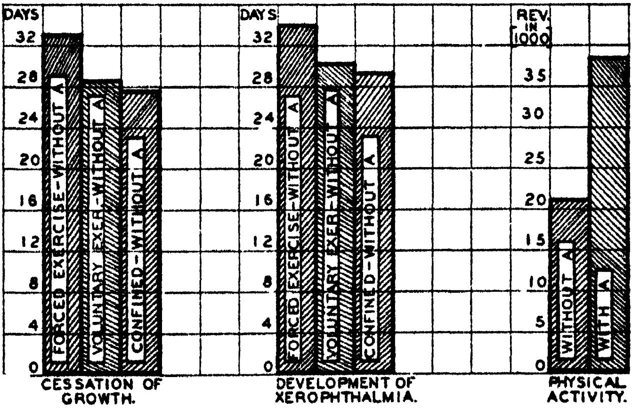

Fig. 4 The effect of exercise on the average time required for the cessation of growth and for the development of the symptoms of xerophthalmia among young rats receiving a diet deficient in vitamin A. This figure also shows the relative activity (as measured by rotation of the cage wheel) of comparable groups of young rats, of which the animals of one group received daily 2 U.S.P. units of vitamin A throughout the experiment, while the animals of the other group received no vitamin A supplement during the first part of the experiment but received 2 U.S.P. units of vitamin A daily during the last 35 days.

an average, 33 days to bring the animals of the forced exercise group to a constant body weight, while similar animals in the confined cages required an average of 27.5 days. Likewise, an average of 34 days was required to produce definite symptoms of xerophthalmia among the animals of the forced exercised group, while those of the confined group required only 29.6 days. These data are not in agreement with those obtained by Keith and Mitchell ('23). However, the two experimental procedures can hardly be considered comparable. It seems possible that the vitamin A requirement of a young animal may be more definitely related to its rate of growth than to its physical activity. It has been frequently observed in this laboratory in connection with our regular vitamin A assays that those animals which make the most rapid rate of growth during the depletion period also are the first to develop the symptoms of vitamin A deficiency. Hence, it appears logical to assume that the confined animals which made the greatest increase in body weight and in consequence developed the greatest demand for the vitamin, should show the earliest symptoms of the deficiency.

Data are also presented in figure 4 to show the effect of vitamin A intake on the physical activity of two comparable groups of animals while being maintained in the freely rotating cages. These data represent the average number of voluntary rotations made by the animals of the two groups. It is to be noted that those animals which had been previously depleted of their vitamin A reserves made a total of 22,000 revolutions during the experimental period, while those animals which received 2 U.S.P. units of vitamin A daily made a total of 38,000 revolutions in a similar period.

It was also observed that a number of the animals composing the voluntary exercise group did not register an appreciable number of revolutions during their first week in the rotating cages. But with increased familiarity with their cage-quarters, all animals of this group showed greater inclination to rotate the wheel. In the case of those animals receiving 2 U.S.P. units of vitamin A daily (fig. 5), this increase in activity continued throughout the experimental period but did not equal the activity previously observed among young animals receiving the breeding colony diet. Those animals which received the vitamin A deficient diet without supplement showed weekly increases in the number of rotations until the end of the fourth week when slight evidences of vitamin A deficiency were becoming apparent. During the following week, these animals showed a marked decrease in activity which continued through the seventh week, although each animal of this group received 2 U.S.P. units of vitamin A daily beginning with the sixth week of the experiment. During the eighth, ninth and tenth week, all animals of this group showed a definite return to activity. In spite of this rapid return to activity the average number of rotations made by this group of animals was always considerably less than the number of rotations made during corresponding weeks of the experiment by the animals in a comparable group which had received the vitamin supplement

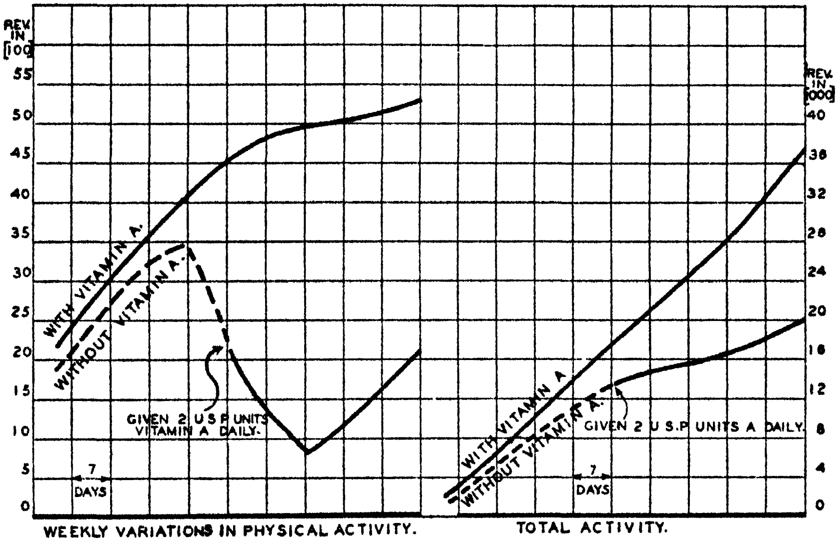

Fig. 5 The average weekly variation in physical activity and also the total activity of two groups of young rats maintained under comparable conditions in all respects except as to their vitamin A intake.

from the beginning of the experiment. These findings further emphasize the importance of an adequate vitamin A intake in the promotion of vigorous physical activity.

SUMMARY

Experiments carried out with carefully selected groups of rats as experimental subjects yielded data to show that under comparable conditions of experimentation less food was consumed, smaller increases in body weight were made and less severe symptoms of vitamin A deficiency developed when the animals were forced to exercise than when allowed to exercise voluntarily or when confined in the usual type of cage.

Although animals maintained under the condition of forced exercise exhibited the greatest efficiency of food utilization, they voided the greatest number of fecal pellets, thus indicating the beneficial effect of exercise on intestinal motility.

Animals maintained under the condition of voluntary exercise and which received daily allotments of vitamin A were more active physically than litter mates which did not receive the vitamin A supplement, further indicating a relationship between completeness of diet and physical activity.

LITERATURE CITED

BLACKLOCK, D. B. 1924 Lack of exercise as a determining factor in the epidemiology of beri beri. Brit. Med. J., no. 3311, pp. 1046–1047.

KEITH, M. HELEN, AND H. H. MITCHELL 1923 The effect of exercise on vitamin requirement. Am. J. Physiol., vol. 65, pp. 128–138.

YOSHIUE, S. 1922 Über den Einfluss der Funktion auf den Verlauf der Avitaminosen. Biochem. Z., Bd. 134, S. 375–380.

THE RELATION OF DIETARY FAT TO THE THIAMIN REQUIREMENTS OF GROWING RATS[1, 2]

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THREE FIGURES

(Received for publication December 14, 1938)

There still appears to be some question as to the effect of increased amounts of fat in the diet on the thiamin requirements of animals. As Evans, Lepkovsky and Murphy ('34) have pointed out, Funk ('14) early suggested that the severity of thiamin deficiency symptoms could be correlated with the amount of carbohydrate in the diet. There is now ample evidence that thiamin is related to the metabolism of pyruvic acid.

The first report to show definitely that rats can be maintained with greatly reduced thiamin intakes when the ration contains high levels of fat was that of Evans and Lepkovsky ('28). Salmon and Goodman ('37) have studied the question recently and a review of the literature is included in their paper.

We became interested in this problem for two reasons. First, we had available a basal ration which was complete in all nutrients except thiamin. This ration, described by Arnold and Elvehjem ('38), gives normal growth in rats when supplemented with adequate thiamin. Second, we felt that much

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[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

[2] Presented in part at the thirty-second annual meeting of the American Society of Biological Chemists, March, 1938 (Stirn and Arnold, '38).

[3] Now with U. S. Standard Products, Woodworth.

[4] Wilson and Company fellow.

more uniform results would be obtained if the sucrose in the basal ration was replaced by the fat on an isocaloric basis. This procedure would not alter the ratio of the remaining constituents of the diet.

EXPERIMENTAL

Composition of rations. Table 1 gives the composition of basal ration 112 as well as the percentage composition of the modifications containing varying amounts of fat. In these modifications one-fourth (ration 112a), one-half (ration 112b), three-fourths (ration 112c) and all of the sucrose (ration 112d) have been isocalorically replaced by fat. It must be mentioned that the autoclaved yeast and the autoclaved peanuts supply about 6.5% fat to ration 112. The figure for the amount of fat in the modifications of ration 112 does not include the fat supplied by these ingredients.

TABLE 1

Composition of diets showing procedure in replacing sucrose by isocaloric amounts of fat

CONSTITUENTS	R. 112		R. 112a		R. 112b		R. 112c		R. 112d	
	parts	*%*	*parts*	*%*	*parts*	*%*	*parts*	*%*	*parts*	*%*
Sucrose	60	60	45	49.2	30	36.2	15	20.1		
Fat			6.5	7.1	13	15.7	19.7	26.2	26	39.4
Purified casein	18	18	18	19.7	18	21.7	18	24.1	18	27.3
Factor W ≅ liver extract	2	2	2	2.2	2	2.4	2	2.7	2	3
Autocl. peanuts	10	10	10	10.9	10	12.1	10	13.5	10	15.2
Autocl. yeast	4	4	4	4.4	4	4.8	4	5.4	4	6.1
Salts 1	4	4	4	4.4	4	4.8	4	5.4	4	6.1
Paper pulp	2	2	2	2.2	2	2.4	2	2.7	2	3
Total	100[1]	100	91.5	100	83	100	74.7	100	66	100
Caloric ratio sucrose/fat	100% sucrose		75/25		50/50		25/75		100% fat	

[1] Vitamin A and D supplied separately to all animals as a concentrate.

The animals. Albino rats were placed on experiment at about 25 days of age at a weight of 40 to 50 gm. The animals were kept in individual cages equipped with wire screen bottoms (two meshes to the inch). Distilled water was obtained from inverted bottles suspended on the side of each cage. Growth records were obtained from weekly weighings.

Procedure. The rats were depleted of their thiamin reserves by restriction to ration 112 until the symptoms of polyneuritis appeared. Occasionally the rats had limited their food intake to the extent that cures were not obtained when rations high in fat were offered to the rats. The number of such animals was very small and with more careful observation such animals were rarely encountered.

Comparison of growth of rats fed ration 112 supplemented with thiamin chloride and ration 112d without added vitamin. The composite growth curve of three rats (male) which had been polyneuritic and which had then been fed thiamin chloride are shown in figure 1. The amount of thiamin chloride fed (15 micrograms daily) insured good growth. Similar growth resulted with the three animals (male) which had been brought out of the polyneuritic condition by the isocaloric substitution of fat for carbohydrate (ration 112d) in the diet. In this instance coconut oil was used. The animals which received the vitamin supplemented ration 112 gained 20.1 gm. per week for the following 6 weeks. The animals fed ration 112d (coconut oil) gained 20.5 gm. per week for the comparable period. Three rats (female), grew, as would be expected, at a somewhat slower rate (fig. 1). The average for the three was 14.5 gm. per week for the 6-week period follow-

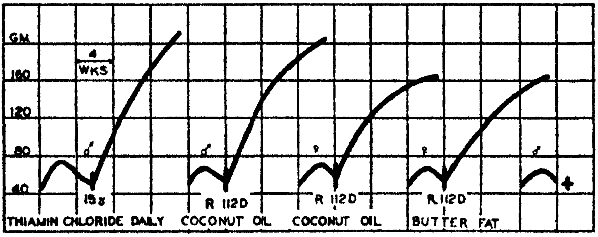

Fig. 1 Growth records of rats fed ration 112 to deplete their thiamin reserves and then fed either ration 112 supplemented with 15 micrograms of thiamin chloride daily or ration 112d using either coconut oil or butterfat to isocalorically replace the sucrose component of the ration.

ing the change in rations. The use of butterfat rather than coconut oil as in the previous groups made no appreciable difference in the growth rates of five rats (female) (fig. 1). The curve shown in figure 1 shows a gain of 14.0 gm. per week for the 6-week period following the change in diets. The individual growth records of the rats are not significantly different from the composite growth records as given in the figure. The three control animals fed ration 112 died at the end of the fourth week on the diet.

These results satisfied us that a vitamin sparing action could easily be demonstrated on a diet which was adequate in all the nutritive factors needed by the rat aside from thiamin.

Results of partial replacement of the carbohydrate by fat. Because of the extreme efficacy of fat in alleviating polyneuritis and producing growth when the sucrose was replaced by isocaloric amounts of fat in ration 112, we were interested in determining the effect of the partial replacement of carbohydrate by fat. The animals were fed ration 112 until all exhibited severe symptoms of thiamin deficiency. The animals were then divided into four groups and placed on ration 112a, 112b, 112c and 112d respectively in which coconut oil partially or completely replaced the sucrose.

The growth of the animals in this experiment is shown in figure 2. Ration 112a, which contained 7.1% coconut oil, was able to alleviate the polyneuritic symptoms. The recovery period, however, was much prolonged. Growth was poor and amounted to only 5.0 gm. per week for the 7-week experimental period. Ration 112b, which contained 15.7% fat, was more effective in curing the symptoms and allowed an average growth of 5.3 gm. per week. Ration 112c, which contained 26.2% fat, produced results similar to those obtained on ration 112d. The former ration allowed growth of 10.3 gm. per week for the 7-week period while the animals fed the latter ration gained 15.4 gm. per week for the same period.

The animals fed ration 112d, which contained cottonseed oil, coconut oil or olive oil (fig. 2), were continued for 27 weeks on the high fat rations. The female animals in these groups had normal estrous cycles as exhibited by vaginal smears. At the close of the experiment the females weighed 180 to 200 gm. and the males about 220 to 260 gm. The animals were apparently normal in every respect.

The effect of various levels of synthetic tricaproin. The results obtained with synthetic tricaproin are most interesting. The polyneuritic symptoms slowly disappeared when the animal was shifted to ration 112a (7.1% synthetic tricaproin).

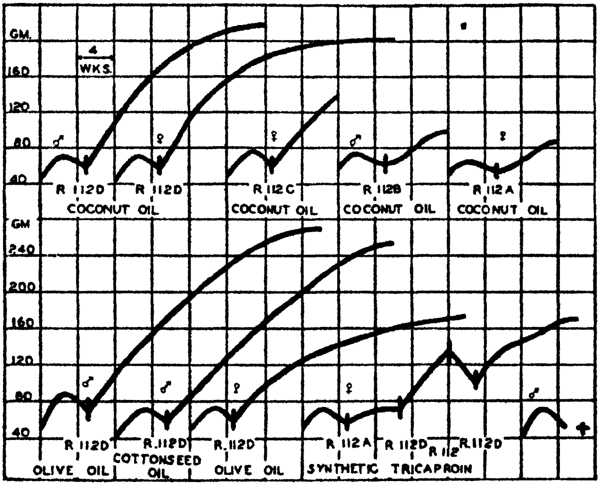

Fig. 2 Growth records of individual rats fed ration 112 to deplete their thiamin reserves and then fed coconut oil at levels which isocalorically replaced one-fourth (ration 112a), one-half (ration 112b), three-fourths (ration 112c) or all (ration 112d) of the sucrose in the diet. Also growth records of individual rats depleted as before and then fed ration 112d where olive oil, cottonseed oil or synthetic tricaproin (two levels) isocalorically replaced the sucrose.

Growth, however, was poor (2 gm. per week) for the 6-week period. This animal was then fed ration 112d (39.4% tricaproin) and immediately gained in weight. During the 5-week period the gain was 10.2 gm. per week. Ration 112 was then substituted for 3 weeks during which time there was a loss in weight of 4.5 gm. per week. Although this animal had been on experiment for 19 weeks, when again fed ration 112d (tricaproin) it made an average gain of 7 gm. per week for the following 10 weeks.

This demonstrated that the requirement for thiamin was markedly reduced by as little as 7.1% synthetic tricaproin. It further showed that an animal could be made to gain or lose in weight merely by varying the amount of fat and sucrose in the thiamin deficient ration.

The effect of autoclaving on the thiamin sparing action. A study was made to determine whether the activity of natural fats would be decreased by autoclaving the fats for 10 hours at 15 pounds pressure. The fats were autoclaved in shallow pans at a depth of about ¼ inch.

TABLE 2

Weekly growth response of polyneuritic rats during the first 3 weeks on ration 112d (autoclaved coconut oil) and on ration 112d (autoclaved coconut oil) supplemented with thiamin chloride

RATION	AVERAGE GAIN IN WEIGHT			APPROXIMATE TIME FOR RECOVERY FROM POLYNEURITIC SYMPTOMS
	1st week	2nd week	3rd week	
	gm.	*gm*	*gm.*	
Ration 112d (six rats)	23.5	17.7	16.5	3 days
Ration 112d + 15 micrograms thiamin chloride per day (six rats)	30.6	20.0	17.3	5 hours

The results in table 2 show the growth response of polyneuritic animals cured on ration 112d (autoclaved coconut oil) as compared with autoclaved coconut oil supplemented with 15 micrograms of thiamin chloride per animal per day. It was quite evident that the vitamin gave a supplementary effect only during the first week. This effect gradually decreased until at about the sixth week both groups made equal gains. Animals which received the vitamin supplement were apparently free from polyneuritic symptoms within 4 to 6 hours after the administration of the vitamin and immediately began to consume the fat ration. Animals on the unsupplemented fat rations regained their appetites more slowly.

The above animals were sacrificed at the end of the third week for determinations of the cocarboxylase content of the livers and brains.[5] The tissues of rats fed the high fat diets were compared with similar tissues from polyneuritic animals and animals which were fed the stock colony ration. The results are summarized in table 3. It is evident that the livers from the rats fed the high fat rations are very similar to the tissues from polyneuritic rats, while those getting the normal ration are definitely higher. The brains do not show such great differences.

TABLE 3

Cocarboxylase content of the livers and brains of rats fed ration 112, ration 112d or the colony stock ration

RATION	COCARBOXYLASE CONTENT	
	Liver	Brain
	cmm. CO_2	*cmm. CO_2*
Colony stock ration	536	381
Ration 112		
(rats polyneuritic)	187	170
Ration 112d	205	318

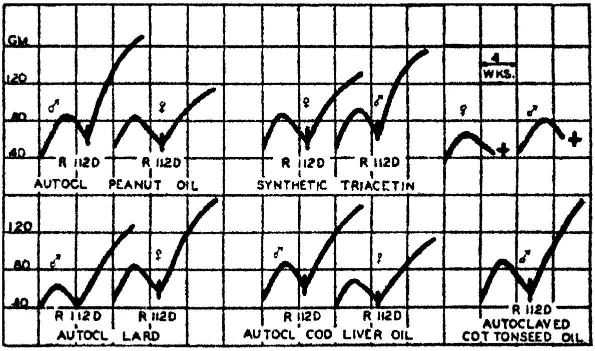

Fig. 3 The growth records of individual rats fed ration 112 until polyneuritic symptoms appeared and then fed ration 112d in which synthetic triacetin, autoclaved peanut oil, lard or cod liver oil isocalorically replaced the sucrose component of the ration.

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[5] The cocarboxylase values were kindly determined by Mr. M. A. Lipton according to the procedure of Lipton, Potter and Elvehjem ('38).

Differences with various diets. The response of polyneuritic animals to ration 112d using various other autoclaved natural fats which included peanut oil, lard, cod liver oil and cottonseed oil is shown in figure 3. Synthetic triacetin was also tested. All fats effectively alleviated the polyneuritic symptoms and supported growth. A limited number of animals in some groups which had gained weight the first week still showed mild polyneuritic symptoms. These symptoms usually disappeared within 8 days. Of this group the three females on peanut oil, lard and cod liver oil, and the male on cottonseed oil showed severe symptoms at the end of the first week yet the growth response was 10, 22, 8 and 13 gm. respectively. The fact that growth resulted in the presence of polyneuritic symptoms indicated that fats produced their effect independently of thiamin since the vitamin cured polyneuritis in 4 to 6 hours.

DISCUSSION

The results presented in this report are, we believe, not entirely at variance with the results presented by the earlier investigators. Since Gregory and Drummond ('32) were not able to control their experiments with pure thiamin, it is most likely that these workers had a multiple deficiency in their diets which was masked by the use of yeast concentrates. Kemmerer and Steenbock ('33) concluded that fats had no vitamin sparing action since the livers and muscle tissues of the rats fed the high fat diets contained no more thiamin than did the rats fed the vitamin low basal ration. The data presented in table 3 corroborate these findings. The livers of the rats fed the high fat diets are significantly lower in cocarboxylase than the livers from rats fed the colony stock ration. The evidence, therefore, shows that while the animals are unable to build up or maintain their thiamin reserves they are still able to obtain energy from the metabolism of fat for growth purposes. The data in table 3 add further evidence for the view that the basal ration is extremely low in its thiamin content although we are unable completely to rule out the possibility that it does carry traces of the vitamin.

In certain respects the results presented here do not agree with those of Salmon and Goodman ('37). These investigators observed 100% incidence of polyneuritis in rats fed a thiamin low ration which contained 40% of cottonseed oil, pecan oil, linseed oil, soft lard, beef fat, olive oil or butterfat. The average time for the onset of the polyneuritis symptoms in the rats fed the latter three fats was apparently the same as that on the low fat ration (p. 484, table 3, 37 days). The nutritive ratio of the 40% fat diet used by these workers was approximately 5.7 : 1 which corresponds to a low fat diet with about 15% protein. It is possible that the diets fed by these investigators were low in factors other than thiamin since growth responses also resulted when autoclaved yeast was added to the diet (p. 483, fig. 1). It may be noted that when the autoclaved yeast supplemented to a high fat diet was increased sufficiently no cases of polyneuritis were observed (p. 484, table 3). It is also seen that even in the presence of adequate thiamin (p. 483, fig. 2, curve 5) growth was less than 2 gm. per day during the first 8 weeks on experiment. We have previously found (Arnold and Elvehjem, '38) that even autoclaved yeast cannot always be depended upon to supply adequate amounts of the members of the vitamin B complex other than thiamin.

Whipple and Church ('36) have suggested that thiamin is necessary for fat synthesis because animals on a thiamin low diet have a decreased fat reserve while the reserve is increased upon the addition of the vitamin. The same conclusions were arrived at as a result of studies on the respiratory quotient (Whipple and Church, '37). It is not possible from any present work to determine whether thiamin is specifically necessary for fat synthesis. However, we would like to point out that the decreased fat stores can be explained in a slightly different way in the light of our results. In thiamin deficiency the rat preferentially metabolizes fat as opposed to carbohydrate. This wipes out the fat reserves.

In the presence of thiamin the rat is able to metabolize carbohydrate normally both for energy and for fat synthesis which allows for an accumulation of fat. The fact that rats will eat a ration high in fat when showing polyneuritic symptoms while the intake of a vitamin low-sucrose ration is reduced, indicates that dietary deficiencies influence food choice. Barelare, Holt and Richter ('38) have demonstrated the increased appetite for fat by rats on a thiamin deficient diet through free choice experiments.

SUMMARY

The evidence indicates that when the carbohydrate component of a thiamin-free ration is isocalorically replaced by natural fats or synthetic fatty acid esters the resultant diet will alleviate the polyneuritic syndrome of thiamin deficient rats.

Female rats fed a high fat ration (39.4% fat) grew at good growth rates and had normal estrous cycles.

The livers of rats fed a high fat diet were as low in cocarboxylase content as were those of polyneuritic animals. The brain tissue of rats fed thiamin-deficient high-fat diets retained a greater reserve of vitamin containing coenzyme.

No marked differences have been observed between olive oil, coconut oil, autoclaved peanut oil, autoclaved lard and autoclaved cottonseed oil as judged by the growth records of the rats.

Synthetic fatty acid esters, tricaproin and triacetin, were also effective in alleviating the polyneuritic syndrome of thiamin deficient rats.

LITERATURE CITED

ARNOLD, A., AND C. A. ELVEHJEM 1938 Studies on the vitamin B_1 requirements of growing rats. J. Nutrition, vol. 15, p. 429.

BARELARE, B., JR., L. E. HOLT, JR. AND C. P. RICHTER 1938 Influence of vitamin deficiencies on appetite for particular foodstuffs. Am. J. Physiol., vol. 123, p. 7.

EVANS, H. M., AND S. LEPKOVSKY 1928 Sparing action of fat on the antineuritic vitamin. Science, vol. 68, p. 298.

EVANS, H. M., S. LEPKOVSKY AND E. A. MURPHY 1934 The sparing action of fat on vitamin B. VI. The influence of the levels of protein and vitamin G. J. Biol. Chem., vol. 107, p. 429.

FUNK, C. 1914 Studien über Beriberi. XI. Die rolle der Vitamine beim Kohlenhydratstoffwechsel. Zeit. f. physiol. Chem., Bd. 89, S. 378.

GREGORY, E., AND J. C. DRUMMOND 1932 A study of fat metabolism with special reference to nutrition on diets devoid of fat. Z. Vitaminforsch., vol. 1, p. 257.

KEMMERER, A. R., AND H. STEENBOCK 1933 A study of the sparing action of fats on the vitamin B content of animal tissues. J. Biol. Chem., vol. 103, p. 353.

LIPTON, M. A., V. R. POTTER AND C. A. ELVEHJEM 1938 The relation of vitamin B_1 to cocarboxylase. Biochem. J., vol. 32, p. 474.

SALMON, W. D., AND J. G. GOODMAN 1937 Alleviation of vitamin B deficiency in the rat by certain natural fats and synthetic esters. J. Nutrition, vol. 13, p. 477.

STIRN, F. E., AND A. ARNOLD 1938 The relation of dietary fat to the vitamin B_1 requirement of growing rats. J. Biol. Chem., vol. 123, p. cxvii.

WHIPPLE, D. V., AND C. F. CHURCH 1936 The composition of growth induced by vitamin $B(B_1)$. J. Biol. Chem., vol. 114, p. cvii.

——— 1937 The effect of vitamin B_1 on the respiratory quotient. J. Biol. Chem., vol. 119, p. ciii.

THE HEAT PRODUCTION AND BLOOD AND URINE CONSTITUENTS AFTER ADMINISTRATION OF d-LYSINE MONOHYDROCHLORIDE TO THE DOG

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Although the literature dealing with amino acids is quite large, there yet remain considerable gaps in our knowledge of the basic physiological properties of many of these compounds. To cite briefly only one phase of our incomplete information, we may point out the fact that five of the ten amino acids now generally conceded to be nutritional essentials (Rose, '38), isoleucine, methionine, tryptophane, lysine and threonine have never been individually tested to determine their effect upon heat production.

Inasmuch as we have had available adequate quantities of lysine, arginine and histidine, it seemed highly desirable to add to the existing data by comparatively detailed studies of the rates of metabolism of these acids and their effects upon the heat production of normal animals. Incidentally such experiments would seem to be a very valuable preliminary to maintenance of an animal by completely parenteral administration of amino nitrogen.

Although lysine has long been recognized as an essential amino acid, little is known concerning its intermediary metabolism. Dakin ('13) has shown that it produces neither extra glucose nor β-hydroxy butyric acid in the phlorhizinized dog. Rapport and Beard ('28) considered arginine responsible for most of the specific dynamic action of the basic amino acid fraction of proteins, but owing to the high cost of lysine they were unable to make a direct test of this substance. So far as we are aware, no one has attempted such a study since that time.

METHODS

Dogs thoroughly trained for metabolic experiments were used in this investigation. They were maintained throughout on a constant diet and fasted at least 16 hours previous to each experiment. The experiments were all performed at room temperatures of 25 to 30°C., which is within the range of thermal neutrality for the dog.

Several determinations of basal metabolic rate and also of blood amino and urea nitrogen were obtained in a control period preceding the administration of the amino acid. Blood urea and amino nitrogen levels, as well as heat production, were determined hourly during a 6-hour experimental period following lysine administration. Heat production was measured by the Tissot-Haldane method, urea by the manometric urease method of Van Slyke ('27) and amino nitrogen by the manometric method of Van Slyke ('29). Urine collections were begun and terminated by catheterization. The control period was of approximately 2 hours duration, while the experimental period lasted about 6 hours. Both control and experimental samples were analyzed for total nitrogen by the Kjeldahl method, for urea by the manometric urease method of Van Slyke ('27) and for amino nitrogen by the manometric method of Van Slyke ('29). The amino nitrogen of the urine was determined directly after removal of urea and also, in some cases, upon the phosphotungstic acid precipitates.

In three experiments amytal anesthesia was used. However well trained a dog may be, there are times when slight movements will occur which will increase the heat production slightly but appreciably. This does not occur in dogs under deep amytal anesthesia and the specific dynamic action is unchanged (Eaton, Cordill and Gouaux, '36). Thus, even a small increase in heat production would represent a true specific dynamic action.

Four experiments were performed with glycine to serve as a check on the methods used and to provide a basis for comparison with the lysine studies. The amino acids for intravenous injection were dissolved in about 60 cc. of water, sterilized by heat and injected at a constant rate into a saphenous vein during a period of approximately 10 minutes.

RESULTS

Blood constituents. Average values for blood amino nitrogen following the administration of glycine and lysine are presented in table 1. It will be seen that the blood amino nitrogen level 1 hour after intravenous injection of glycine was very much higher than at the same time following lysine injection. This difference is due principally to the larger amount of nitrogen given in the glycine experiments. Even after allowing for the difference in the amount of nitrogen, however, we find that the lysine is eliminated from the blood somewhat more rapidly than the glycine during the first hour when the concentration is high, and somewhat less rapidly than the glycine during the third, fourth and fifth hours when the concentration is approaching the control figure.

TABLE 1

Average values for blood amino nitrogen and blood urea nitrogen after administration of glycine or lysine

NUMBER OF EXPERIMENTS	AMINO ACID GIVEN	METHOD OF ADMINISTRATION	AMINO NITROGEN GIVEN	MILLIGRAMS PER 100 CC. OF BLOOD						
				Control period (hours before injection)		Experimental period (hours after injection)				
				1	0.5	1	2	3	4	5
			Amino nitrogen							
			gm.							
4	Glycine	Intravenous	1.796	9.8	9.5	29.4	13.9	11.2	11.6	10.8
2	Lysine	Intravenous	1.296	9.7	9.5	19.7	13.5	12.0	12.0	12.5
6	Lysine	Intraperitoneal	1.466	9.1	9.1	19.6	14.2	12.5	11.4	11.5
			Urea nitrogen							
4	Glycine	Intravenous	1.796	11.6	12.0	15.7	19.3	19.9	17.6	15.9
2	Lysine	Intravenous	1.296	12.9	13.6	13.1	13.8	13.1	12.2	11.5
6	Lysine	Intraperitoneal	1.466	12.2	12.2	12.0	13.0	14.7	14.8	14.2

From an examination of table 1 it is also evident that lysine is metabolized more slowly than glycine, since the blood urea nitrogen increase after lysine is very small in contrast to the large increase after giving glycine. These two results taken together indicate that, when a relatively large amount of lysine is injected directly into the blood stream, the compound is quite rapidly excreted and only a small fractional portion is utilized. This fact is strikingly apparent from the data in table 2. In the two intravenous experiments an average of only 276 mg. of the amino nitrogen (equivalent to 1.8 gm. of lysine monohydrochloride) were retained by the animal to undergo metabolism. For this reason we resorted to intraperitoneal injections in the hope that the compound would be absorbed more slowly and hence be retained in the body for a sufficient period of time to allow more nearly complete utilization. This hope was partially realized. The blood amino nitrogen value during the second hour is probably better maintained by the latter method. There is also a slight but significant increase in the blood urea nitrogen using this technic. Lysine, in this case, was being deaminized slightly faster than the kidneys eliminated the urea.

TABLE 2

Average urea and amino nitrogen excretion after administration of glycine or lysine

NUMBER OF EXPERIMENTS	AMINO ACID	METHOD OF ADMINISTRATION	AMINO NITROGEN GIVEN	EXCESS URINE UREA NITROGEN	EXCESS URINE AMINO NITROGEN	
					Total	Diamino
			gm.	*gm.*	*gm.*	*gm.*
4	Glycine	Intravenous	1.790	0.714	0.563	
2	Lysine	Intravenous	1.296	0.524	1.020	
1	Glycine	Intraperitoneal	1.790	0.780	0.356	
6	Lysine	Intraperitoneal	1.466	0.347	0.629	0.437

It is an interesting coincidence that in the dog lysine is metabolized slowly and is readily excreted when its concentration in the blood rises appreciably and that (Doty and Eaton, '37) the rate of absorption from the gastro-intestinal tract (as determined on rats) also proceeds at a slow rate as compared with other amino acids. If the same relative absorption rate is found for dogs, one might be tempted to infer the presence of a mechanism for supplying the organism with this amino acid at a rate at which it can be utilized efficiently.

Urinary constituents. The data summarized in table 2 are largely self-explanatory. The excess urine urea nitrogen after an intravenous administration of lysine was greater than after an intraperitoneal injection. This does not, however, indicate a more rapid rate of urea formation in the former case. Thus an average of 79% of the intravenously administered amino nitrogen appeared in the urine as such, while the extra urea nitrogen excreted, if derived from the lysine, would account for 40% of the amino acid nitrogen injected. In other words, the extra urinary urea nitrogen was two times as large as the amino nitrogen retained by the dog. It seems obvious that the marked diuretic action of the lysine, when it was placed directly in the blood stream, resulted in the washing out of considerable urea in excess of that arising from metabolism of lysine. This is further borne out by the blood urea nitrogen figures from table 1. It will be noted that the blood urea nitrogen actually fell (after intravenous injection of lysine) about 2 mg. per cent during the period of the experiment. Hence the urine values following the intraperitoneal injection must be regarded as more nearly representative of the urea formed in the metabolism of lysine.

When this amino acid was given by the latter method, an average of 43% of its nitrogen was excreted into the urine as amino nitrogen. Extra urea was equivalent to 24 of the remaining 57%. The amount of extra ammonia was in all cases too small to be of any significance. Furthermore, the extra total nitrogen as determined by the Kjeldahl method had a tendency to be slightly lower than the sum of the extra urea and amino nitrogen values. There was, then, at least 33% of the intraperitoneally administered nitrogen which could not be accounted for. That this discrepancy was due, in any considerable degree, to delayed absorption of the lysine from the peritoneal cavity does not seem probable in view of the findings regarding blood amino nitrogen. In table 1 it can be seen that the amino nitrogen values of the blood rose rapidly during the first hour and by the end of the third or fourth hours had subsided to a rather constant level only slightly above the control level. We feel justified, therefore, in assuming that the greater part of the lysine reached the blood stream, that about one-fourth of its nitrogen was transformed to urea nitrogen, that there was at least a temporary storage of about one-third of its nitrogen and that the remainder of its nitrogen was excreted as amino nitrogen.

One other point deserves special mention. In the six intraperitoneal experiments the amino nitrogen of the urine was estimated both after removal of urea and following precipitation with phosphotungstic acid. There is considerable discrepancy between the two values. It would appear that nearly one-third of the excess amino nitrogen of the urine appeared in some form other than lysine. Further studies of this phenomenon will soon be instituted.

TABLE 3

Heat production after administration of glycine or lysine

DOG NUMBER	WEIGHT	CONTROL PERIOD		EXPERIMENTAL PERIOD			EXCESS URINE UREA N	AMINO ACID METABOLIZED	S.D.A.
		Average B.M.R	Basal urine urea N	Amino acid used	Average metabolic rate	Urine Urea N			
				Intravenous injection					
	Kilos	*Cal./hr.*	*mg./hr.*	*gm.*	*Cal./hr*	*mg./hr.*	*mg.*	*m. Mols.*	*Cal./m.Mol*
1	11.4	16.70	89.8	G 9.6	19.11	219.6	687	49.06	0.245
1	11.4	18.92	104.7	G 9.6	21.17	236.0	659	47.07	0.239
1	11.3	18.15	87.0	G 9.6	20.87	248.5	832	59.43	0.230
1	11.0	18.43	33.7	G 9.6	20.98	163.8	677	48.36	0.264
Average									0.244
1	10.8	17.88	78.1	L 8.45	18.40[1]	170.3	458	16.36	0.159[1]
				Intraperitoneal injection					
1	11.1	17.81	47.5	L 8.86	17.75	99.0	243	8.68	—0.034
				Intraperitoneal injection (amytal anesthesia)					
3	11.4	17.39	142.0	L 9.56	17.55	205.4	324	11.57	0.069
2	9.8	15.95	65.5	L 9.56	15.87	143.2	388	13.84	—0.029
3	10.3	15.81	40.9	L 9.56	15.71	126.2	438	15.65	—0.051
Average									—0.004

[1] Rise in second hour period only; dog restless.

Heat production. The specific dynamic action of glycine is in good agreement with the values previously reported (Eaton, Cordill and Gouaux, '36, and Wilhelmj and Mann, '30). In only two of the experiments on lysine was there any increase in heat production (table 3). In one of these the increase was so slight as to be insignificant. One other experiment showed a rise which was appreciable, however, only in the second hourly period when the dog displayed some restlessness. Thus this experiment cannot be regarded as indicating any dynamic action. We can only say that if lysine does possess a specific dynamic action, it is too low to be measured by the usual methods.

Although all attempts to administer lysine by stomach tube resulted in vomiting, we cannot say that lysine showed any toxic effects. Dog 1 received lysine on four occasions extending over a period of 19 days. That no serious kidney damage occurred is indicated by a rather constant and low blood urea nitrogen value throughout this period. The dog appeared in excellent health throughout. Our results do not support the findings of Newburgh and Marsh ('25) that lysine is definitely nephrotoxic.

SUMMARY

Lysine is metabolized much more slowly than glycine. It is readily excreted by the kidneys if present in the blood in large quantities.

So far as we are able to determine, lysine does not produce a specific dynamic action.

LITERATURE CITED

DAKIN, H. D. 1913 Studies on the intermediary metabolism of the amino acids. J. Biol. Chem., vol. 14, p. 321.

DOTY, J. R., AND A. G. EATON 1937 Metabolism of the basic amino acids. I. Rates of absorption in ratio of the monohydrochlorides of d-lysine and l-histidine. J. Biol. Chem., vol. 122, p. 139.

EATON, A. G., S. C. CORDILL AND J. L. GOUAUX 1936 The specific dynamic action of glycine intravenously administered to nephrectomized dogs. J. Nutrition, vol. 12, p. 113.

NEWBURGH, L. H., AND P. L. MARSH 1925 Renal injuries by amino acids. Arch. Int. Med., vol. 36, p. 682.

RAPPORT, D., AND H. H. BEARD 1928 The effects of protein split-products upon metabolism. III. Further investigation of the fractionated protein hydrolysates and of amino acids, and their relation to the specific dynamic action of the proteins. J. Biol. Chem., vol. 80, p. 413.

ROSE, W. C. 1938 The nutritive significance of the amino acids. Physiol. Rev., vol. 18, p. 109.

VAN SLYKE, D. D. 1927 Determination of urea by gasometric measurement of the carbon dioxide formed by the action of urease. J. Biol. Chem., vol. 73, p. 695.

———— 1929 Manometric determination of primary amino nitrogen and its application to blood analysis. J. Biol. Chem., vol. 83, p. 425.

WILHELMJ, C. M., AND F. C. MANN 1930 The influence of nutrition on the response to certain amino acids. I. The effect of fasting. Am. J. Physiol., vol. 93, p. 69.

THE HEAT PRODUCTION AND BLOOD AND URINE CONSTITUENTS AFTER ADMINISTRATION OF d-ARGININE MONOHYDROCHLORIDE TO THE DOG

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Although many papers dealing with arginine have appeared from time to time, there seem to be very few data in the literature concerning the effect of administered arginine on the heat production, the blood constituents and the nitrogenous constituents of the urine.

Thompson ('05 a and b) studied the nitrogenous constituents of the urine both after feeding and after injecting arginine. In most of the experiments the greater part of the arginine nitrogen could be recovered from the urine, chiefly in the form of urea. There was also some increase in the ammonia of the urine amounting to the equivalent of about 10% of the arginine nitrogen. Upon feeding arginine, none appeared in the feces, and in fact there was a decrease in fecal nitrogen on the experimental days. Arginine, ornithine and putrescine could not be detected in either urine or feces. The intravenous injection of arginine produced no toxic effects.

Rapport and Beard ('28) in a single experiment administered 5 gm. of arginine carbonate to a dog. They observed an increase in the heat production and in the urinary nitrogen. Kiech, Luck and Smith ('31) studied the rate of catabolism of arginine injected into rats. They found this substance to be catabolized rapidly. Ornithine, if formed, must have been utilized almost as rapidly, since at no time could more than

11% of the arginine nitrogen be unaccounted for as either arginine or urea.

The present study is an attempt to extend the earlier work quoted above and to add further data concerning the rate of utilization of arginine.

METHODS

With but few exceptions the plan of experiment and the methods of analysis have already been described (Doty and Eaton, '39). Urinary amino nitrogen was determined directly on the urine (after removal of urea) and also upon the silver arginine precipitate prepared according to Block ('34). Ammonia was determined by the method of Folin.

In two of the experiments reported here, 9.9 gm. of the arginine monohydrochloride were administered intravenously, and in two cases the same quantity was given by the oral route. In another experiment in which the amino acid was injected intravenously the changes in blood and urine nitrogen constituents were noted, but changes in heat production were not measured. For intravenous administration, the material was dissolved in about 60 cc. of water, sterilized by heat and injected into a saphenous vein at a constant rate during a period of 10 minutes. An attempt to administer a water solution of 20 gm. of the arginine by stomach tube was not successful since the dog lost at least a large part of the solution by vomiting. Our former experience had demonstrated that oral administration of amino acids in water solution often resulted in vomiting. Because of this fact, in experiment 18 we dissolved the arginine in a warm 2% solution of agar agar, allowed the agar to congeal and then forced the dog to swallow this solid material. A later test showed that the dog would retain 10 gm. of the amino acid in water solution when given by stomach tube. In experiment 19 this method of administration was used. The results of experiments 18 and 19 are entirely comparable, the only difference being that in the presence of the agar the compound is apparently absorbed at a somewhat slower rate.

RESULTS

Blood urea and amino nitrogen. The results of blood analyses are shown in table 1. The control values for both urea and amino nitrogen are well within normal limits for the dog. The amino nitrogen values in the two intravenous experiments show a marked similarity to the figures which Kiech, Luck and Smith ('31) obtained by analyzing the entire carcasses of rats after injection with arginine. Our amino nitrogen values tend to return to control levels in a shorter time, but the dosage of amino acid per unit of body weight was somewhat lower. With oral administration changes in the curves are those to be expected when the rate of absorption is considered (Doty and Eaton, '38). After intravenous injections of arginine the blood urea values began rising promptly and attained their highest levels within 3 hours. Throughout the remainder of the experimental period the figures showed only a slight tendency to return to the control levels. The maxima are reached with both glycine and arginine at about the same

TABLE 1

Blood amino nitrogen and blood urea nitrogen values after administration of 9.9 gm. of arginine monohydrochloride

DOG NO.	EXPERIMENT NO.	METHOD OF ADMINISTRATION	MILLIGRAMS PER 100 CC. OF BLOOD						
			Control period (hours before administration)		Experimental period (hours after administration)				
			1	0.5	1	2	3	4	5
			Amino nitrogen						
4	15	Intravenous	10.1	10.0	14.3	11.5	9.4	9.0	9.3
4	16	Intravenous	10.1	10.0	15.8	11.6	10.2	9.6	9.4
5	20	Intravenous	10.4	10.4	20.8	14.8	11.6	11.2	11.2
4	18	Oral (in agar)	8.1	8.0	8.0	8.4	9.0	9.4	9.4
4	19	Oral	8.3	8.3	9.1	10.2	10.5	9.6	8.7
			Urea nitrogen						
4	15	Intravenous	13.6	13.7	17.8	20.5	22.6	21.0	18.0
4	16	Intravenous	13.3	13.3	16.8	19.8	22.8	22.2	21.3
5	20	Intravenous	6.1	6.3	11.6	15.1	17.4	17.1	16.1
4	18	Oral (in agar)	16.8	16.8	17.8	19.2	20.6	21.8	23.4
4	19	Oral	18.4	18.5	20.3	22.4	23.4	24.2	24.8

time but with glycine the decrease in blood urea is somewhat more rapid. The failure of blood urea nitrogen values to return to control levels soon after the termination of the specific dynamic action makes the interpretation of the heat production rise more difficult.

Urinary nitrogen. Unchanged arginine appeared in the urine in all experiments. Reference to table 2 shows that the quantity excreted after intravenous administration is fairly large and of about the same order as found in the case of glycine when it is given intravenously (Doty and Eaton, '39; Eaton, Cordill and Gouaux, '36). As would be expected, the excretion following oral administration was very small. By this method the concentration of blood amino nitrogen never increased more than 2 mg. %. The figures for arginine excretion as given in table 2 were obtained by analyses of the silver precipitates. Figures so similar were obtained in each case from direct analyses of the urines after removal of urea that we may infer, as did Thompson ('05 b), that if ornithine is formed it is not excreted in appreciable quantities. It should be noted, in this connection, that both amino groups of ornithine are removed in the Van Slyke analysis and, hence, the amino nitrogen values after removal of urea would be much higher than those of the silver precipitates if any appreciable amount of ornithine were excreted. This was not the case. Kiech, Luck and Smith ('31) have inferred from their experiments on rats that ornithine, if formed, is probably almost as rapidly catabolized as is arginine itself.

TABLE 2

Heat production and urinary nitrogen after administration of 9.9 gm. of arginine monohydrochloride

EXPERIMENT NO.	ARGININE EXCRETED	S.D.A. CAL.	EXCESS NON-ARGININE URINARY N	CAL./GM. OF EXCESS URINARY N	CAL./M M. METABOLIZED
	gm.		*gm.*		
15 (i.v.)	2.97	4.85	0.226	21.44	0.383
16 (i.v.)	2.88	3.35	0.144	23.26	0.415
18 (oral)	0.54	5.61	0.288	19.48	0.348
19 (oral)	0.63	6.88	0.441	15.60	0.279
Average				19.95	0.356

The values given in table 2 for excess non-arginine urinary nitrogen are obtained by deducting four times the amino nitrogen from the Kjeldahl figures. Since they are in close agreement with the urinary urea nitrogen values the latter are not included in the table. In experiments 18 and 19 urinary ammonia nitrogen was determined. The excess was 74 and 86 mg. respectively, a much lower value than that reported by Thompson ('05 a).

Heat production. Our results prove conclusively that arginine produces a specific dynamic action. There are obvious objections to relating the increase in heat production to the excess urinary nitrogen or in comparing it with the stimulating action of glycine. One difficulty arises from the failure of the blood urea nitrogen to return to control level soon after termination of the specific dynamic action of the arginine. It has been demonstrated, by way of contrast, that an equal quantity of glycine is metabolized rapidly enough so that the blood urea values have returned to the control level in most cases by the end of a 5-hour experimental period (Doty and Eaton, '39; Eaton, Cordill and Gouaux, '36). Since with arginine all of the increase in heat production occurs within the first 4 or 5 hours, it would probably introduce an additional error to collect the urine for longer than the 6-hour period. We have calculated our results on the same basis as for glycine but call the attention of the reader to the fact that these values may be unavoidably higher than the true figures. On this basis arginine causes a much lower absolute rise in heat production than does an equal quantity of glycine. On the other hand, when expressed as Calories per millimol of arginine completely catabolized, the dynamic action of arginine is somewhat higher than that of glycine. One must, of course, make allowance on this basis for the high blood urea nitrogen at the end of the experimental period.

So far as we have been able to find, only a single experiment has been performed on the specific dynamic action of arginine. Rapport and Beard ('28) gave 5 gm. of arginine carbonate to a dog by mouth. They obtained an increase in heat production of 5.7% above basal. If one recalculates their data on the basis used by us, one finds the value of 19.7 Cal./gm. of excess urinary nitrogen. This remarkably close agreement is most striking when one considers all of the unavoidable error which may creep into metabolic work and also when one realizes that different analytical methods were used.

We are not, as yet, prepared to discuss the specific dynamic action of arginine and its cause in relation to the possible participation of the guanidine group, ornithine or any other substance derived from the arginine molecule. We hope to have at our disposal a sufficient quantity of ornithine in the near future to extend our studies to this important substance.

SUMMARY

Upon either intravenous or oral administration of arginine monohydrochloride to the dog there is a prompt and long sustained rise in the urea nitrogen of the blood. There is an excretion of unchanged arginine in the urine reaching considerable proportions in the intravenous experiments. Arginine is metabolized somewhat more slowly than glycine.

Arginine exhibits a specific dynamic action, which calculated upon the basis of the amount administered, is less than that of glycine but calculated upon the basis of Calories per millimol deaminized is probably slightly higher than that of glycine.

Ornithine, if formed, does not appear in the urine in appreciable quantities.

No toxic effects of arginine were noted.

LITERATURE CITED

Block, R. J. 1934 The determination of the basic amino acids in small quantities of protein by the silver precipitation method. J. Biol. Chem., vol. 106, p. 457.

Doty, J. R., and A. G. Eaton 1938 Rate of absorption of the basic amino acids as a function of the amount fed. Am. J. Physiol., vol. 123, p. 53.

——— 1939 Metabolism of the basic amino acids. II. Studies on the heat production and blood and urine constituents after administration of d-lysine monohydrochloride to the dog. J. Nutrition, vol. 17, p. 1.

Eaton, A. G., S. C. Cordill and J. L. Gouaux 1936 The specific dynamic action of glycine intravenously administered to nephrectomized dogs. J. Nutrition, vol. 12, p. 113.

KIECH, V. C., J. M. LUCK AND A. E. SMITH 1931 Studies on arginine. I. The rate of catabolism of arginine in rats, including a method for the determination of arginine in biological material. J. Biol. Chem., vol. 90, p. 677.

RAPPORT, D., AND H. H. BEARD 1928 The effects of protein split-products upon metabolism. III. Further investigation of the fractionated protein hydrolysates and of amino acids, and their relation to the specific dynamic action of the proteins. J. Biol. Chem., vol. 80, p. 413.

THOMPSON, W. H. 1905 a The physiological effects of peptone and allied products. Part VI. The metabolism of arginin. J. Physiol., vol. 32, p. 137.

——— 1905 b The physiological effects of peptone and allied products. Part VII. The metabolism of arginin. J. Physiol., vol. 33, p. 106.

A STUDY OF THE ASCORBIC ACID INTAKE REQUIRED TO MAINTAIN TISSUE SATURATION IN NORMAL ADULTS

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ONE FIGURE

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A number of attempts have been made to estimate the vitamin C requirement of human beings. This subject has been recently reviewed by Smith ('38). According to this author, three levels of requirement may be distinguished: the physiologically indispensable, the adequate, and the saturation or luxus consumption values. The comment has recently been made (Current Comment, '38) that "It may well be that the varying results of different laboratories may be brought into harmony if distinction is made between actual requirement and indispensable minimum, the latter being the amount that is sufficient to prevent scurvy and the former also including that quantity of vitamin C necessary for normal functioning of all body processes which may require or be influenced by vitamin C." In the case of substances which are readily excreted when taken in excess of bodily needs the assumption is often made that a desirable allowance is that amount which produces maximum retention. Although there is at present no clear evidence that it is desirable to maintain the tissues is a constant state of saturation with respect to vitamin C, it is obvious that the daily requirement under ordinary circumstances cannot be greater than the minimum amount which will maintain such a state of saturation.

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These experiments were designed to determine the smallest ascorbic acid intake which would maintain the tissues in a fully saturated state with respect to vitamin C and thus to set an upper limit to the amount of ascorbic acid which the adult can use under normal conditions of health and activity.

EXPERIMENTAL

Subjects

Seven normal adults, five women and two men, served as subjects for this study. Table 1 gives information concerning the heights, weights and ages of the subjects at the beginning of the experiment and their basal metabolic rates expressed in calories. Subjects F and G gained several pounds in the course of the experiment, subject B usually lost 2 or 3 pounds at the beginning of an experimental period, and the others tended to maintain their original weights or to fluctuate only slightly.

TABLE 1

The age, sex, height, weight and basal metabolism of each subject

SUBJECT	AGE	SEX	HEIGHT IN INCHES	INITIAL WEIGHT IN POUNDS	BASAL METABOLISM IN CALORIES PER 24 HOURS
A	38	Female	61	140	1340
B	37	Female	62	130	1390
C	23	Female	64½	115	1435
D	22	Female	68¾	135	1160
E	34	Female	70½	167	1700
F	24	Male	72¼	160	1670
G	23	Male	72¾	159	1820

Because of the possible effect of activity on vitamin C metabolism, severe physical exercise was avoided, and the subjects recorded any unusual activity.

Methods

Diet. The basal diet used in the study was a modification of that used in a previous experiment (O'Hara and Hauck, '36). The following foods were used ad libitum: a whole wheat cereal with added wheat germ, butter, brown and refined sugar, Ry Krisp, whole wheat and white flour, pecans, cooking fat, rice and coffee. Each subject had an egg each day, and eggs were used to a limited extent in cooking, for example in making cookies. The following foods were used in weighed amounts either because they contained traces of vitamin C or because a certain intake was considered advisable to insure the adequacy of the diet: 100 gm. of canned beets plus 10 gm. of juice, 100 gm. of canned diced carrots plus 10 gm. of juice, 100 gm. of cooked dried prunes plus 25 gm. of juice, 100 gm. of canned pears plus 50 gm. of juice, 60 gm. of evaporated milk, 100 gm. of ground beef and 60 gm. of Cheddar cheese. The average amount of vitamin C furnished by the basal diet was approximately 10 mg. daily, as estimated from indophenol titration. An ascorbic acid supplement [1] dissolved in a small amount of water was given daily at breakfast. The 400 mg. test dose was divided equally between the morning and noon meals.

Urine collections. Since in fifty-eight out of sixty-four periods, urine titrations were made twice daily, in the morning and early evening, day and night specimens were collected separately. The day sample included the urine collected at the first voiding after breakfast and throughout the day. Night collections included urine voided from the evening through the first voiding after rising. Each subject collected this last specimen at approximately the same time each morning.

Each urine specimen was divided into two equal parts, one of which was placed in a brown bottle and was acidified immediately with 10% by volume of 2 N sulfuric acid containing 2% of metaphosphoric acid. This portion was used for the estimation of vitamin C excretion, whereas the remaining portion was kept for the pH determination. Both portions were stored in the refrigerator. Preliminary observations indicated that specimens of urine preserved in this manner lost little vitamin C in 24 hours. In nine observations, the loss ranged from 0 to 6%, with a mean loss of less than 2%.

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[1] Synthetic ascorbic acid (Hoffman La Roche) was used except during the period when subjects C, D and E received a 25 mg. supplement, at which time Merck's Cebione tablets were used.

Determination of vitamin C in urine. The vitamin C content of the urine samples was determined by titration with a buffered solution of the indicator, sodium 2,6-dichlorobenzenone-indophenol,[2] standardized every other day against a known solution of ascorbic acid.

The indicator was run quickly from a microburette into a measured portion of the acidified, refrigerated urine. Either 2 ml. or 5 ml. aliquot portions were used depending upon the concentration of the urine and the amount of vitamin C it was expected to contain.

All acid solutions used for preserving the urine, the solutions of indicator and of ascorbic acid for standardizing the dye were made up with water redistilled from glass.

Determination of urinary pH. The pH of 24-hour specimens of urine was determined colorimetrically, using the La Motte Universal pH outfit.

Plan of the experiment

Before beginning any experimental period, the subjects were saturated by giving them large doses of vitamin C, usually in the form of the juice of 4 to 8 oranges daily for 3 days in addition to the usual diet. The urine was collected and titrated on the day preceding the experimental period, to be sure that the subject was saturated. With the exception of a few instances preceding saturation periods when supplements of 200 mg. of ascorbic acid were given, the urinary excretion on this day did not fall below 190 mg. of ascorbic acid. In the majority of instances the excretion of ascorbic acid on the day preceding the experimental period was between 200 and 300 mg.

The response to a 400 mg. test dose following a 4- or 5-day period during which a 200 mg. supplement of vitamin C was given daily, was taken as the criterion for complete saturation. At least three such periods were studied for all subjects.

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[2] Eastman Kodak Company.

Similar test doses were given following 6-day periods on various lower levels of ascorbic acid intake and the responses were compared with those obtained when the subject was known to be saturated. If the response to the test dose equaled or exceeded the lowest response by the same subject following a period when the supplement was 200 mg. daily, the subject was considered to be saturated. Thus each subject provided his own individual standard for comparison.

In earlier experiments it was noted that if a subject had been previously saturated, the level of excretion of ascorbic acid was approximately constant after the second or third day on a constant intake. It was assumed therefore that a 6-day period would allow sufficient time for adjustment to a given level of intake, and with few exceptions, this was the period used.

The results presented here represent a total of sixty-four experimental periods, or from seven to twelve periods for each subject, covering a total time of from 5 to 17 months.

RESULTS AND DISCUSSION

Requirement to maintain tissue saturation

The values reported as ascorbic acid excretion represent total indophenol-reducing substances present. The enzymic method suggested by Tauber and Kleiner ('35) was used to determine what effect the basal diet might have on the urinary output of reducing substances other than vitamin C. In twenty-one determinations, for six subjects, there were only three instances in which the non-specific reducing capacity of the urine exceeded 3 mg. per day. The average value was 1.6 mg. As judged by this method, therefore, the amount of reducing substance other than vitamin C was too small to affect the interpretation of results. In any case, the amount of non-specific reducing substance in the urine could not have exceeded the total excretion of indophenol-reducing substance in a previous experiment (O'Hara and Hauck, '36) in which the subjects excreted 9 to 12 mg. daily during the fourth week on the basal diet only.

The urinary responses to the 400 mg. test dose following saturation periods during which a daily supplement of 200 mg. of ascorbic acid was taken in addition to the basal diet are shown in table 2 (last column). It will be noted that subjects A and G gave no individual response above 249 mg., and that subjects B and C gave none lower than 240 mg. Individual differences in behavior have been observed by other authors, notably Wachholder and Hamel ('37) who relate them to differences in kidney threshold.

Subjects A, D, E and F showed a fairly regular steplike increase in response to the test dose with increasing levels of intake, until a value characteristic of saturation was reached (fig. 1 and table 2). Subject C's responses to the test dose were somewhat more irregular, her excretion following the 60 mg. level being 158 mg. as compared to 179 mg. following the 35 mg. level of intake. The low response of this subject following the 110 mg. level was probably due to diarrhea. No explanation is apparent for the variable behavior of subject G in response to the test doses.

The responses of subject B to test doses following levels of intake from 35 to 85 mg. were remarkably similar. Since marked differences between the 35 and 60 mg. levels were apparent when the experimental period was continued for 2 weeks instead of 6 days, it appears that the 6-day experimental period was not long enough for this subject to show small differences in tissue saturation. Note that this subject tended to give a high response to the test dose following saturation periods. Such behavior would be expected if the kidney threshold was low, but too few data are available on the plasma level of vitamin C for this subject to indicate whether this is the explanation.

The data in table 2 indicate that a minimum intake of between 70 to 85 mg. of ascorbic acid was necessary to maintain tissue saturation in subjects C and D under the conditions

TABLE 2

Twenty-four hour excretion of ascorbic acid in response to the test doses, following periods on various levels of ascorbic acid intake

SUBJECT	DAILY INTAKE OF ASCORBIC ACID IN MILLIGRAMS												
	10	35	60	70	80	85	90	95	100	110	125	135	210
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
A				133	191	204	201		266				222, 227, 248
B	141	191, 107[1]	189[1]	204	203	201			254				242, 274, 306
C		179	158[2]	239		253				196[3]			240, 269, 281
D		117	215[3]	215		247				251			228, 256, 275
E		62	140[2]			177		201, 207	232, 204[1]				200, 210, 228, 278
F									160[1]	178	210	245[4]	222, 210, 232
G			107			172			139	216, 237[4]	167	225[4]	209, 241, 249

[1] Subject had been on this level of intake for 14 days instead of 6 days before the test dose.

[2] Subject had been on this level 4 days only, before the test dose.

[3] Subject suffered from diarrhea on this day.

[4] Determination made on pooled 24-hour specimens.

of this experiment. Subject A required between 90 and 100 mg., subject B between 85 and 100 mg. and subject E about 95 to 100 mg., just to maintain tissue saturation. The male subjects, F and G, apparently required more vitamin C, although they were of approximately the same size as subject E. Subject F required between 125 and 135 mg. of vitamin C daily to maintain saturation, and although subject G's responses were more variable, his requirement appeared to be between 110 and 135 mg. When calculated on the basis of the lower figure in each case, the requirement per kilogram of body weight ranged from approximately 1 mg. for subject D to 1.6 mg. for subject F. This would represent approximately 60 to 96 mg. for an adult weighing 60 kg. If the upper figure is used as a basis for calculation, the variation is from approximately 1.2 to 1.7 mg. per kilogram or from 72 to 102 mg. for an adult weighing 60 kg. Thus, as a recent review has pointed out (Smith, '38) the least amount of vitamin C which will maintain tissue saturation is several times the amount estimated as the physiologically indispensable minimum.

According to Smith ('38) ". . . . a comparison of the absolute and relative values for different ages suggests that vitamin C requirements are related both to body weight and to metabolic rate, the effect of the latter being the more pronounced." For this reason, basal metabolic rates of the subjects are recorded (table 1). In general there is a rough correspondence between the basal metabolism in calories and the amount of vitamin C required to maintain saturation. It is possible that there may be a more direct relation between total energy metabolism and vitamin C requirement.

It is of interest that the amount of vitamin C necessary just to maintain tissue saturation, as determined in these experi-

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Fig. 1 Urinary excretion of ascorbic acid for seven subjects on various levels of ascorbic acid intake. In most instances where data were available for more than one period on the same level of intake, one period only is represented. The saturation period shown for each subject is the one in which the response to the test dose was lowest. x indicates that the urine specimen was not available for analysis; y, a day on which the subject had diarrhea; z, a period during which no ascorbic acid supplement was given.

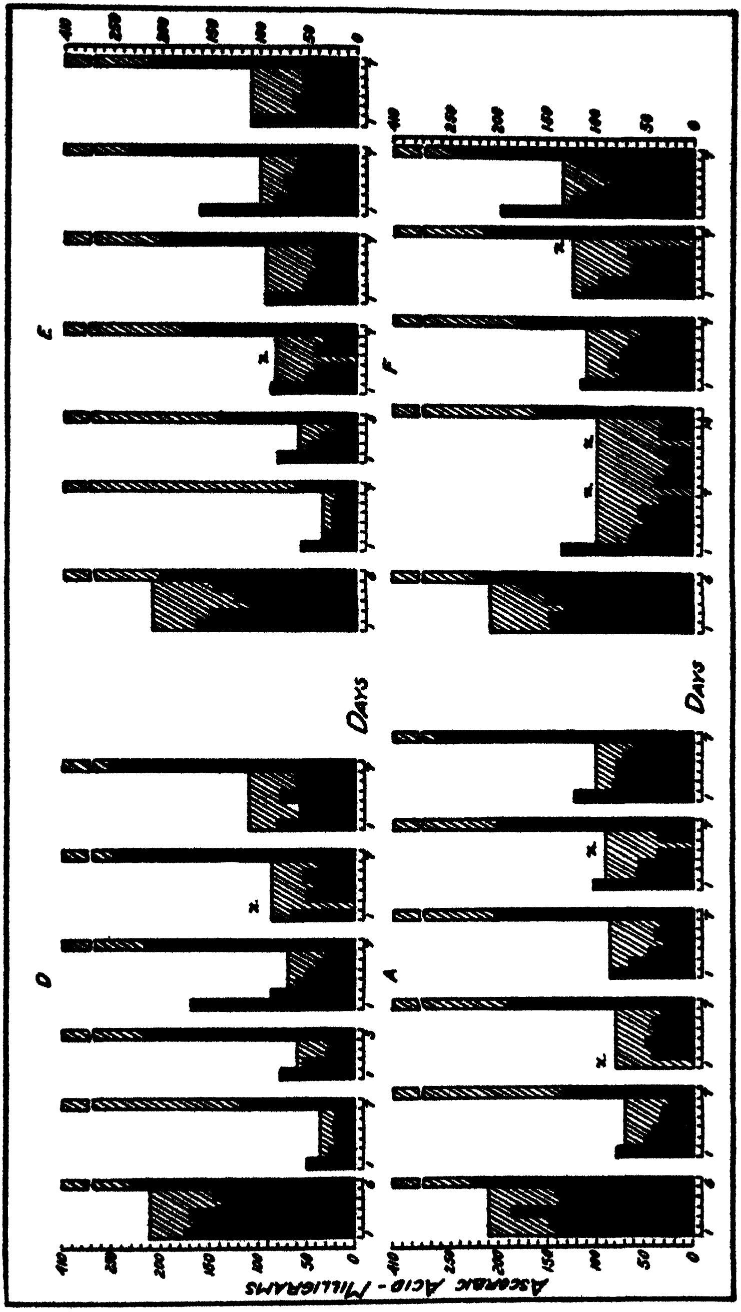

Figure 1 a

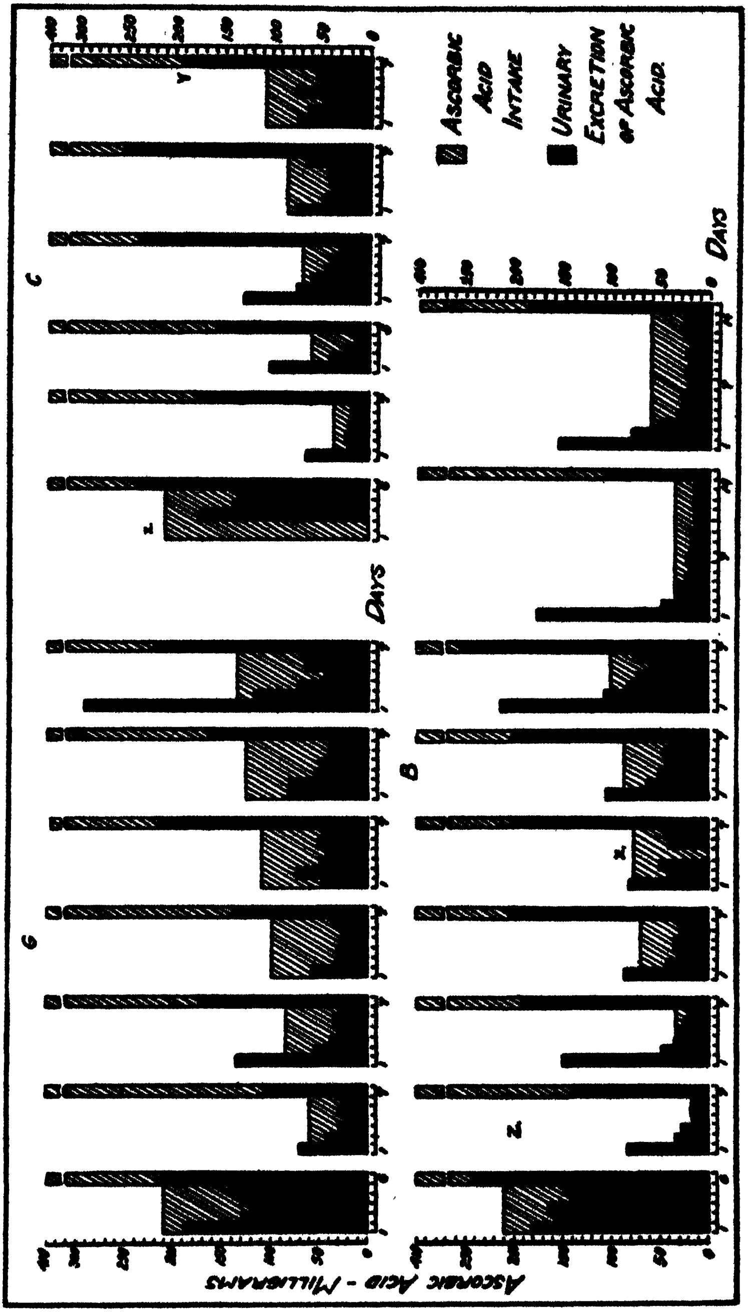
Ascorbic Acid - Milligrams
0
50
100
150
200
250
300
G
B
C
x
y
z
Days
Days
Ascorbic Acid Intake
Urinary Excretion of Ascorbic Acid

Figure 1 b

ments is very similar to King's estimate[8] of a desirable allowance for normal persons, arrived at by a different method. Standard guinea pigs are protected from gross scurvy by an intake of 0.5 mg. of ascorbic acid per day, but twice this amount is required for normal tooth structures if no other stress is brought to bear. Larger intakes are necessary to permit good protection against injury produced by bacterial toxins (Swanson, Sigal and King, '36). If toxin injections are made at the 1 mg. level, animals show marked injury to the tooth structure. In contrast, animals receiving 3 mg. per day with the same amount of toxin, show practically no tooth lesions. In human beings, such data as are available indicate that about 15 to 25 mg. per day are essential barely to protect from scurvy. If the requirement for tooth growth in human beings is analogous to that of guinea pigs, 50 mg. would permit the normal development of teeth if no unusual stress occurs. To provide good protection against the injury produced by infection or bacterial toxin, an intake of 80 to 100 mg. per day would be required. On this basis, King expressed the opinion that 100 mg. of vitamin C per day is a desirable allowance for normal persons of all ages, with the exception that this allowance should be increased by 50 mg. for pregnant and lactating women.

On the basis of the present studies, one may assume that such an allowance would permit a relatively high degree of tissue saturation in normal adults under ordinary conditions.

It must be borne in mind that these experiments involved vitamin C supplements as synthetic ascorbic acid, in addition to a basal diet which was distinctly acid forming. The median urinary pH for the various subjects was as follows: for F 5.9, for D and G 5.6, for B 5.5, for A 5.4, for C 5.3 and for E 5.0. In five of the seven cases, the mean, median and theoretical mode were the same. The effect of the reaction of the diet on excretion, storage and requiremnt of vitamin C is still unsettled (Hawley et al., '36; Goddard and Preston,

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[8] King, C. G. 1938 Paper given before Am. Home Econ. Assoc., Pittsburgh, and personal communication.

'38). Preliminary experiments in this laboratory[4] indicate that there are individual differences in the effect of potential acidity or alkalinity of the diet on urinary excretion of vitamin C. This is in accord with the observations of Goddard and Preston ('38).

Factors affecting urinary excretion of vitamin C

Exercise. Although these experiments were not designed to study the influence of such conditions as exercise and environmental temperature upon the apparent requirement for vitamin C, some incidental observations of this sort have been made. For example, following two periods in which the the daily intake of vitamin C was 110 mg. subject G excreted 216 and 237 mg. when a 400 mg. test dose was given. During a third period with a similar intake, the subject played baseball frequently. The response to the test dose following this period was only 156 mg. Since this subject's behavior with respect to vitamin C output was somewhat more variable than that of the other subjects studied, one cannot say with certainty that this variation was due to unusual exercise. However, increased use or disappearance of ascorbic acid following violent exercise has been reported (Hamel, '37).

TABLE 3

Effect of environmental temperature on the urinary excretion of vitamin C

DATES	TEMPERATURE (°F.)			DAY OF EXPERIMENT						RESPONSE TO TEST DOSE
	Mean minimum	Mean maximum	Mean	1	2	3	4	5	6	
				mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Aug. 5–11	67	86	77	119	71	46	52	32	31	182
Sept. 11–17	49	65	55	161	84	70	76	63	61	231

High environmental temperatures. Since the results obtained during the period from October to June could not be duplicated during a hot period in August, it is believed that high environmental temperatures affect the urinary excretion of vitamin C. The figures for urinary output of vitamin C obtained with the same subject during two periods when the daily intake was 100 mg. will serve to illustrate (table 3).

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[4] Unpublished data.

In this connection, the excretion of vitamin C in sweat has been noted by Lilienfeld, Wright and MacLenathen ('36) and Bernstein ('37). Further studies by Wright and MacLenathen[5] have resulted in the conclusion that while vitamin C is excreted in the sweat, it is doubtful that large amounts of this substance are lost in that manner. Martini and Torda ('37) have demonstrated that high temperatures decrease the ascorbic acid content of guinea pig tissue. Whether the lower urinary excretion during the period when the environmental temperature was high is due merely to a relatively larger excretion through the skin, or whether it is indicative of a true lowering of tissue reserves is not clear. Whatever the cause, the results obtained during hot weather differed from those obtained during the fall, winter and spring when the indoor temperature was relatively constant.

Drugs. The subjects were instructed to refrain from the use of drugs during the experiment, since the effect on vitamin C metabolism of certain preparations which are rather commonly used is either not known or in dispute. However, in the course of these experiments, a physician prescribed a medication containing atropine for subject A, and she took it during 1 week of the experiment. The daily excretion of vitamin C was greater when atropine was taken, as compared with similar periods preceding and following the use of the drug, but the response to the test dose was much lower, indicating that the subject was less well saturated. Wright[5] has observed a similar reaction when atropine was administered.

SUMMARY

A method is described for estimating the minimum intake of ascorbic acid which will just maintain the tissues in a state of complete saturation.

Of seven subjects whose requirements have been studied by this method two required between 70 and 85 mg. of ascorbic acid to maintain tissue saturation, three required between 85 and 100 mg. and two required more than 100 mg. of ascorbic acid per day.

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[5] Wright, I. S. Personal communication.

On the basis of body weight, the range in requirement to maintain complete saturation for these subjects was from about 1 to 1.6 mg. per kilogram per day.

Incidental observations on the pH of the urine, the effects of exercise, high environmental temperature and atropine are recorded.

LITERATURE CITED

BERNSTEIN, R. E. 1937 Excretion of vitamin C in sweat. Nature, vol. 140, p. 684.

CURRENT COMMENT 1938 Human requirement for vitamin C. J. Am. Med. Assoc., vol. 110, p. 1928.

GODDARD, V. R., AND R. A. PRESTON 1938 Effect of diet on vitamin C output. J. Home Economics, vol. 30, p. 482.

HAMEL, P. 1937 Ueber die vitamin C-Bilanz des Menschen: II Mitteilung Belastungsversuche zur Bestimmung des täglichen Verbrauches und des Sättigungsdefizits. Klin. Wchnschr., Bd. 16, S. 1105.

HAWLEY, E. E., J. P. FRAZER, L. L. BUTTON AND D. J. STEPHENS 1936 The effect of administration of sodium bicarbonate and of ammonium chloride on the amount of ascorbic acid found in the urine. J. Nutrition, vol. 12, p. 215.

LILIENFELD, A., I. S. WRIGHT AND E. MACLENATHEN 1936 Intramuscular injection of ascorbic (cevitamic) acid and excretion in the sweat. Proc. Soc. Exp. Biol. and Med., vol. 35, p. 184.

MARTINI, E., AND C. TORDA 1937 Die Wirkung der hohen Temperatur auf dem Ascorbinsäuregehalt der Meerschweinchenorgane. Klin. Wchnschr., Bd. 16, S. 1763.

O'HARA, P. H., AND H. M. HAUCK 1936 Storage of vitamin C by normal adults following a period of low intake. J. Nutrition, vol. 12, p. 413.

SMITH, S. L. 1938 Human requirements of vitamin C. J. Am. Med. Assoc., vol. 111, p. 1753.

SWANSON, W. F., A. SIGAL AND C. G. KING 1936 Bacterial toxins and vitamin C in relation to tooth structure. J. Am. Dental Assoc., vol. 23, p. 2089.

TAUBER, H., AND I. S. KLEINER 1935 An enzymic method for the estimation of true vitamin C. J. Biol. Chem., vol. 110, p. 559.

WACHHOLDER, K., AND P. HAMEL 1937 Ueber die Vitamin C-Bilanz des Menschen III Mitteilung Täglicher Bedarf und Güte des Versorgungszustandes nach den Beziehungen des Blutgehaltes zu Ausscheidung und Gewebssättigung. Klin. Wchnschr., Bd. 16, S. 1740.

THE ROLE OF RIBOFLAVIN AND OTHER FACTORS OF THE VITAMIN-B COMPLEX IN THE NUTRITION OF THE PIG [1]

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TWO FIGURES

(Received for publication January 23, 1939)

INTRODUCTION

In a previous publication Hughes ('38) demonstrated that thiamin, riboflavin, nicotinic acid and the filtrate factor (chick anti-dermatitis vitamin or factor 2) were important in the nutrition in the pig. The studies were continued to ascertain more definitely the role of riboflavin, thiamin, the filtrate factor and factor 1 (rat anti-dermatitis vitamin or vitamin B_6) in the growing pig.

EXPERIMENTAL

The conditions of this experiment were similar to those already reported by Hughes ('38). The same, or similar, salt mixtures, cod liver oil, casein, rice screenings, rice bran filtrate, nicotinic acid, thiamin and whey adsorbate were used. Wherever possible pure vitamins were given; where this was impossible vitamin concentrates were employed. The rice screenings used were medium sized pieces of commercial polished rice thoroughly cleaned at the mill. Its content of riboflavin, nicotinic acid and factors 1 and 2 is not known (factor 1, the rat anti-dermatitis factor or vitamin B_6; factor 2, the chick anti-dermatitis factor or filtrate factor); however, all

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[1] Aided by a grant from American Dry Milk Institute, Inc.

grains that have been tested are low in riboflavin and the pellagra-preventive factor (Almquist, Jukes and Newlon, '38; Sebrell, '34).

The protein content of the diets was about 13.75%. Cod liver oil was given each week, 5 cc. until the pigs weighed about 70 pounds, and 10 cc. thereafter. The thiamin and nicotinic acid were given in solution, by mouth, twice each week.

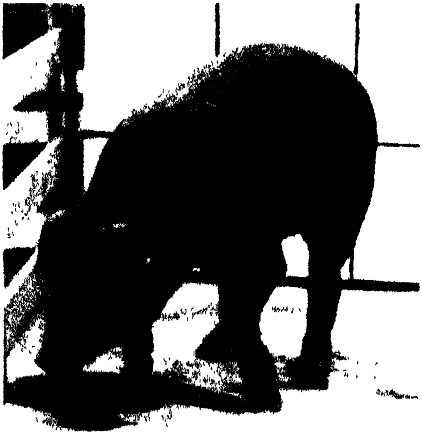

Fig. 1 Showing crippled condition of a pig in lot 2 on a diet deficient in riboflavin. Note the affected ankles, the spreading toes, the bent knees and abnormal positions of the feet.

RESULTS

Except for one pig, which gained very slowly, those in lot 1 (deficient in factors 1 and 2) grew at a moderate rate. Early in the experiment they chewed the sides of the pen and one pig developed an abnormal walk (goose-stepped). The hair and skins of the pigs were normal except for the one that gained very slowly. This pig seemed normal, but the hair was dry. At the close of the experiment a rice-bran filtrate containing largely the filtrate factor and some factor 1 was given, which resulted in an immediate increase in appetite, rate of gain and physical well being.

Those pigs fed a diet deficient in riboflavin (lot 2) gained very slowly, defecated frequently semi-fluid fecal matter, became crippled (see fig. 1) and walked with difficulty. Two of them become bowed in their front legs, turned out at the ankles, stood extremely close at the hocks and the ankles of the hind legs were bent forward excessively. They gained at the rate of 0.64 pound per day; whereas pigs in lot 5, fed the same except their diet contained riboflavin, gained 1.53 pounds per head daily. Three of them were placed in a separate pen after being on the experiment for 72 days and were fed riboflavin in the form of whey adsorbate (1% of the diet) for 21 days. This resulted in improved well being and a slight increase in rate of growth, but did not result in any noticeable improvement in their walking ability.

TABLE 1

Diets fed various groups

FEEDS, CONCENTRATES AND VITAMINS	LOT NUMBERS				
	1	2	3	4	5
Rice screenings, pounds	87	83	83	82	82
Casein, purified, pounds	..	9	9	..	..
Casein, commercial, pounds	9	..	..	9	9
Whey adsorbate, pounds	1	..	1	1	1
Rice bran filtrate,[2] pounds	..	5	5	5	5
Salt mixture, pounds	3	3	3	3	3
Nicotinic acid	14.7 mg.[1]	16.5 mg.[1]	14.0 mg.	..	14.1 mg.
Thiamin	3.7 mg.	4.0 mg.	..	3.5 mg.	3.5 mg.

[1] Milligrams per hundred weight per day.

[2] Fifteen grams of rice bran were utilized in producing 1 cc. of the rice-bran filtrate (Jukes, '37). In diets 2, 3, 4 and 5, 5 pounds of rice-bran filtrate were mixed with the other feeds. The rice-bran filtrate, therefore, represented 5% of the diet.

The pigs in lot 3 (tables 1 and 2) were fed a diet deficient in thiamin. Some thiamin was furnished by the rice, whey adsorbate and the rice-bran filtrate. The pigs ate well for a time, then their appetites decreased. The outward appearance of these hogs was moderately good except that some of them showed a tendency toward leg weakness. Two pigs in this lot died suddenly after being in the experiment 63 and 67 days, respectively, and at autopsy showed very few lesions. One of them showed a cystic bladder with excessively thickened walls. The other pig had a very small spleen. Except for these abnormalities the other organs seemed normal. There was no evidence of pneumonia, of flabby hearts, of abnormal livers or kidneys, and the digestive tract presented no evidence of gastritis, hemorrhage or enteritis. Crystalline B_1 was given as a curative measure to three that remained at the close of the experiment, which resulted in increased appetites and they became definitely more active.

TABLE 2

Average daily gain, feed consumed for 100 pounds of gain and other data for the various groups

Lot number	*Number of pigs*	*Average daily gain in pounds*	*Feed consumed for 100 pounds of gain, in pounds*	*Number of deaths*	*Autopsy and other findings*
1	5	0.83	324	..	Pigs scoured some, one pig gained very slowly, was very unthrifty with watery eyes and dry hair and skin.
2[1]	5	0.64	340	..	They soon showed lack of appetite, all somewhat crippled, scoured some throughout the experiment.
3	5	1.16	350	2	At autopsy one showed a cystic bladder, the other appeared normal except that the spleen was very small.
4	5	1.70	288	..	Scoured some early in test, otherwise normal.
5	5	1.53	293	..	One pig breathed heavily, otherwise normal.

[1] Three pigs were given whey adsorbate after they had been on the experiment 72 days—while they became more thrifty and their physical well being was improved they did not recover from their crippled condition. For the average beginning and final weights see figure 2.

Lots 4 and 5 are of particular interest because those in lot 4 gained faster than those in lot 5. The diet fed lot 4 was not supplemented with nicotinic acid; however, there was apparently enough in the rice-bran filtrate to satisfy their needs. Rice-bran filtrate has been shown by Sebrell and co-workers ('38) to supply the pellagra-preventive (anti-blacktongue) factor. When nicotinic acid was given to the pigs in group 5 they did not gain as fast as those in group 4, which may have been due to an excess of nicotinic acid in this particular diet.

DISCUSSION

The most striking result of the present investigation was the slow growth and the crippled condition of the pigs in lot 2 fed a diet deficient in riboflavin. When riboflavin was added as a curative measure there was an improvement in the physical well being of the animals but they did not recover from their crippled condition, which suggests that permanent injury had taken place due probably to nerve degeneration (Phillips and Engel, '38; Wintrobe, Mitchell and Kolb, '38). That the crippled condition was not some form of rickets was shown by the fact that one animal was killed and showed no evidence of it at autopsy. The bone ash of the humerus was 65%. Riboflavin has thus been shown to be necessary in the nutrition of the pig.

That thiamin is necessary for the pig was shown previously (Hughes, '38). This observation was confirmed by the fact that relatively slow growth was exhibited by pigs in group 3 fed a diet deficient in this factor (fig. 2) as contrasted with lot 5, and that two of the five pigs in this lot died after being on the experiment about 9 weeks. While some of the pigs showed a slight weakness in their legs, they did not exhibit evidence of beriberi paralysis as reported by Mollgaard ('38). The addition of crystalline B_1 to the diet of the three pigs remaining at the close of the experiment resulted in increased appetites, a more thrifty appearance and they became definitely more active.

Those pigs fed a diet deficient in the filtrate factor (factor 2) and factor 1 (vitamin B_6) (lot 1) did not grow normally.

They chewed the sides of their pen, walked poorly and appeared thinner than pigs in lots 4 and 5. Several became crooked in their hocks and one 'goose-stepped' with his hind legs. The addition of rice-brain filtrate (filtrate factor and factor 1) at the close of the experiment caused an immediate increase in appetite and physical well being. Unfortunately we were dealing here with a double deficiency (factor 1 and factor 2).

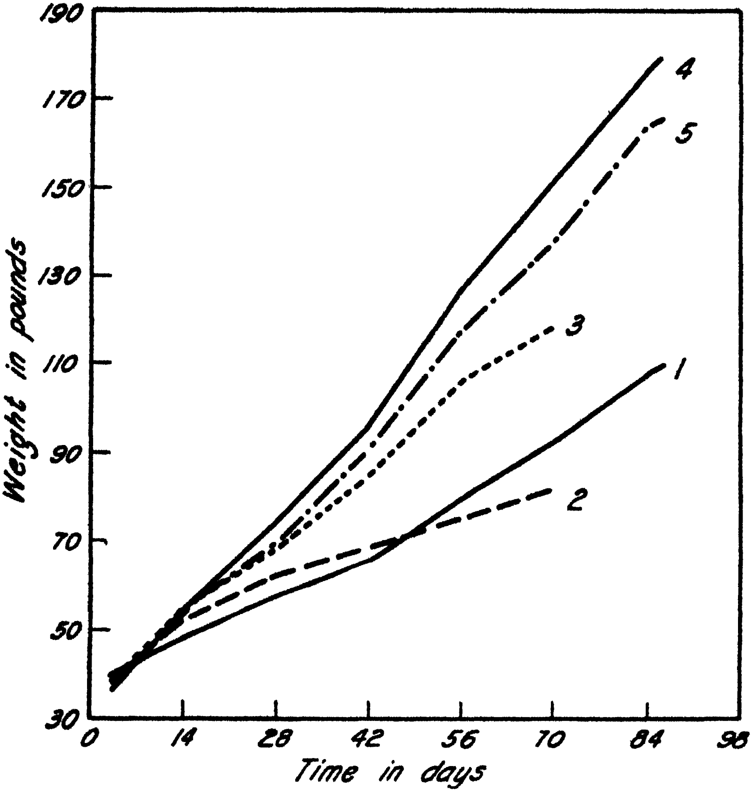

Fig. 2 Growth curves of the various lots. The figures on the graph refer to the lot numbers. The average age of the pigs in the various groups at the beginning of the experiment was 72 days.

The rate of gain of those in lot 4 was exceptionally good. Their growth rate and physical well being can be attributed to the fact that they were receiving a complete diet; that the rice-bran filtrate fed at a 5% level was supplying the needs of the pigs for the filtrate factor, vitamin B_6 and nicotinic acid. The rate of gain (fig. 2) of the pigs in lot 5 was less than that for lot 4. Apparently the addition of about 14 mg. of nicotinic acid per hundred weight each day to the pigs in addition to that received in the rice-bran filtrate in some way slightly depressed their growth. The food utilization of both groups was excellent. It was superior to that of lots 1, 2 and 3.

SUMMARY

Using a purified diet it has been shown that riboflavin is necessary for normal growth and physical well being in the pig. Further evidence is presented showing that thiamin too is necessary for the proper nutrition of the pig.

Feeding a diet without rice-bran filtrate (containing factors 1 and 2) resulted in slow growth, which suggests that one or both of these factors are necessary in the nutrition of the pig.

LITERATURE CITED

ALMQUIST, H. J., T. H. JUKES AND W. E. NEWLON 1938 Feeding chickens. Calif. Agr. Ext. Cir. 108, p. 1.

HUGHES, E. H. 1938 The vitamin-B complex as related to growth and metabolism in the pig. Hilgardia, vol. 11, p. 595.

JUKES, THOMAS H. 1937 Further observations on the assay, distribution and properties of the filtrate factor. J. Biol. Chem., vol. 117, p. 11.

MOLLGAARD, HOLGER 1938 Über störungen im Zentralnervensystem bei der A-Avitaminose des schweines. Tierernährung, Bd. 10, S. 214.

PHILLIPS, PAUL H., AND R. W. ENGEL 1938 The histopathology of neuromalacia and 'curled-toe' paralysis in the chick fed low riboflavin diets. J. Nutrition, vol. 16, p. 451.

SEBRELL, W. H. 1934 Table showing the pellagra-preventive value of various foods. U. S. Pub. Health Reps., vol. 49, p. 754.

SEBRELL, W. H., R. H. ONSTOTT AND D. J. HUNT 1937 The treatment of black tongue with a preparation containing the 'filtrate factor' and evidence of riboflavin deficiency in dogs. U. S. Pub. Health Reps., vol. 52, p. 427.

WINTROBE, MAXWELL M., DAVID M. MITCHELL AND LAWRENCE C. KOLB 1938 Sensory neuron degeneration in vitamin deficiency. Degeneration of the posterior columns of the spinal cord, peripheral nerves and dorsal root ganglion cells in young pigs fed a diet containing thiamin (B_1) and riboflavin but otherwise deficient in vitamin B complex. J. Exp. Med., vol. 68, p. 207.

DETERMINATION OF A CURVE OF RESPONSE TO SYNTHETIC CRYSTALLINE THIAMIN, FOR USE IN THE VITAMIN B_1 ASSAY OF FOODS BY THE RAT-GROWTH METHOD[1]

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TWO FIGURES

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Chemical methods for the determination of vitamin B_1, or thiamin, in foods have not been perfected, and suggested procedures for extracting the vitamin from foods before application of chemical methods are not entirely satisfactory (Williams, '38).

Until suitable chemical methods are devised, it will be necessary to continue the determination of vitamin B_1 in natural foodstuffs by means of a biological method. The accuracy of biological determinations, using either rats or pigeons, may be increased by the use of curves of reference or curves of response, as recommended by Daniel and Munsell ('37) and by Coward ('38). Coward has published such curves for pigeons and for rats using the international standard of reference, an adsorption product on fullers' earth of an extract of rice polishings. There have been a number of reports on the use of the crystalline vitamin B_1 (Heyroth, '36; Kinnersley et al., '35; Waterman and Ammerman, '35), but no work has been reported using a large number of animals fed varying levels of thiamin and giving the resulting curve of response. The work here reported may be of interest to other workers using similar methods and synthetic crystalline thiamin as their standard of reference.

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[1] Published with the approval of the director of the Hawaii Agricultural Experiment Station.

METHODS

The mothers of the experimental rats were fed the following diets during the breeding and lactation periods: whole-milk powder, 400 gm. (or 300 gm. whole-milk powder and 100 gm. skim-milk powder); whole-wheat flour, 1,025 gm.; wheat germ, 40 gm.; salt mixture,[2] 10 gm.; sodium chloride, 10 gm.; soybean meal, 100 gm. One-half of the mothers received the diet containing only whole-milk powder; the other half received the diet containing whole-milk powder and skim-milk powder. On or before the seventh day all litters were reduced to eight or nine young. Records of the weights of the young were made at birth, 7, 10, 14 and 21 days. No differences in weights of rats at birth or at weaning were noted between the litters raised on the two diets. Neither were the reactions to the experimental diet and procedure different for the rats from the mothers fed the two breeding diets.

At 21 days of age, the rats used in the thiamin tests were weaned and placed on the Chase and Sherman diet: vitamin B-free casein, 18%; salt mixture, 4%; cod liver oil, 2%; butterfat, 8%; cornstarch, 53%; autoclaved yeast, 15%. Rats were kept in individual cages with ½ inch mesh wire screens above the bottom of the cages to reduce consumption of excreta. The rats were weighed weekly for the first 2 weeks and daily after that. When the rats had maintained the same weight or lost 1 to 3 gm. each day for 3 or 4 days, the feeding of the thiamin supplements was started and continued for a period of 5 weeks.

Synthetic crystalline thiamin[3] was kept in a thoroughly dry condition by placing it in a brown glass bottle over calcium chloride except for the short period during the weighing process. A 10% alcoholic solution, containing 10 µg. of thiamin in 0.1 ml., was found to be most satisfactory. A 20% alcoholic solution was used for preliminary experiments, but it was believed that some evaporation took place and that greater accuracy could be obtained by using a 10% solution. A fresh solution made each week was placed in a brown glass bottle and kept in a refrigerator, only the small portion needed for each day's feedings being removed at one time. This solution was fed from calibrated pipettes directly into the mouths of the experimental animals. The rats took the solution avidly so that after the first or second feeding there was no danger of losing even the smallest drop.

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[2] Nine parts calcium carbonate and 1 part ferric citrate.

[3] Purchased from the Winthrop Chemical Company.

To supply quantities of thiamin equivalent to 1, 2, 3 and 4 μg. daily (except Sunday), the necessary volumes of the solution were fed three times per week. For 8 and 16 μg. daily (except Sunday), the solution was fed six times per week.

An even distribution of the sexes in each group of rats was desired. Unfortunately, due to mistaken sex identification at the beginning of the experiment, it was necessary to discard some of the rats in several groups even though they responded satisfactorily, in order to equalize the sexes. All the groups receiving 2 to 16 μg. per rat daily had equal division of sexes although the number of pairs in each group varied. In the 1-μg. group there were thirteen females and twelve males, and in the negative control group there were more males than females. The records of 192 rats were used in tables 1 to 4 and figures 1 and 2.

RESULTS

The average weights of the seven groups of rats when placed on the thiamin-free diet at 21 days were very uniform. The mean weight for the 192 experimental rats was 41.1 ± 0.33 gm. with a standard deviation of 4.5 ± 0.23 gm. The rats were depleted of their stores of vitamin B_1 in about 20 days. Animals that did not deplete within 24 to 25 days were found to be unsuitable for experiments, and it was necessary to discard them. When such rats were given supplements, their gains varied greatly from that of the remainder of the group. When they were maintained on the basal diet only, refection apparently took place as their weights in-

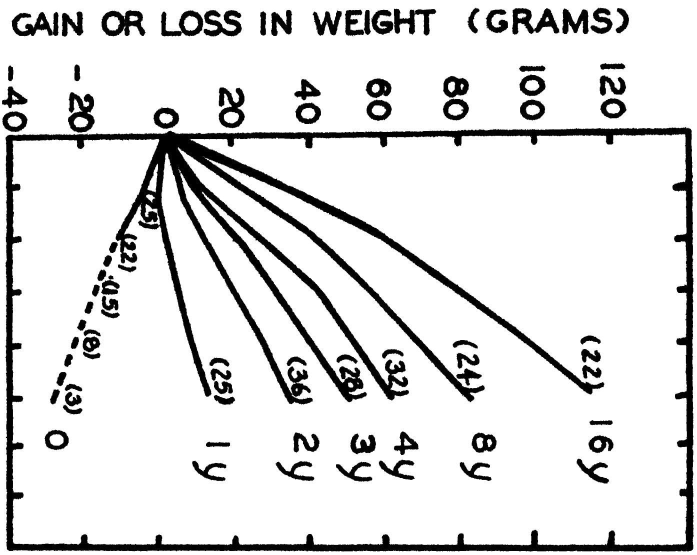

Fig. 1 Average growth response of groups of rats fed varying quantities of synthetic vitamin B_1 (thiamin) after depletion of vitamin B_1 stores. The figures in parentheses show the number of rats used for each test. The broken line indicates the occurrence of the first death in the control group. Spaces on abscissa indicate weeks.

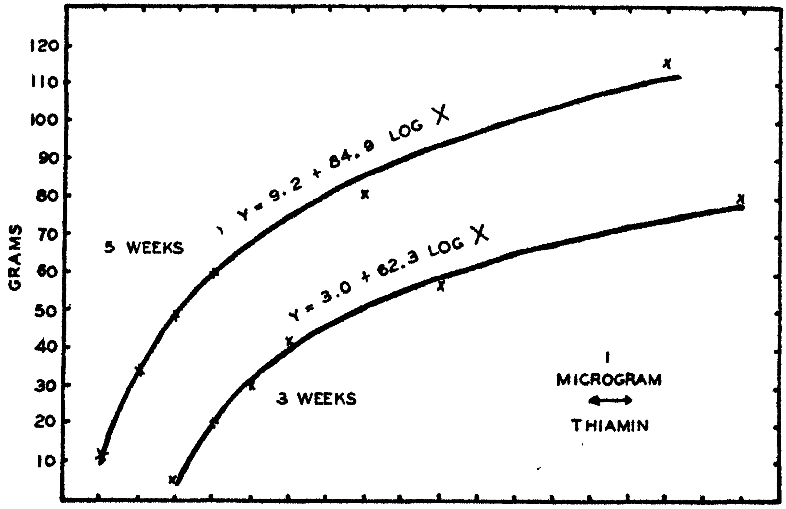

Fig. 2 Calculated curves of response relating gains in weight of rats after 3 weeks and after 5 weeks to doses (in micrograms) of synthetic crystalline thiamin. The crosses indicate actual mean gains in weight of groups of rats fed daily doses of 1, 2, 3, 4, 8 and 16 μg. of thiamin.

creased, even though they previously showed evidence of depletion.

In table 1 are summarized data on the number of rats in each supplement group, the average time for depletion of each group, the average weights of each group at different periods of the experiment and the mean gains in weight of rats in each group after 3 weeks and after 5 weeks of supplement feeding.

The standard errors and 't' values of the mean gains in weight after 3 and 5 weeks of supplement feeding appear in table 2 (Fisher, '36). With the exception of the group of rats receiving the 1 μg. thiamin supplement for 3 weeks, all mean gains in weight for both periods are highly significant as indicated by the 't' values.

TABLE 1

Summary of the results of feeding rats a thiamin-free diet and graded doses of synthetic crystalline thiamin for 3 weeks and for 5 weeks

SUPPLEMENT OF THIAMIN	NUMBER OF ANIMALS	AVERAGE WEIGHTS		AVERAGE DEPLETION PERIOD	FINAL AVERAGE WEIGHTS		MEAN GAINS	
		At weaning	At depletion		At 3 weeks	At 5 weeks	In 3 weeks	In 5 weeks
μg.		*gm.*	*gm.*	*days*	*gm.*	*gm.*	*gm.*	*gm.*
0	25	41	70	21				
1	25	42	71	20	76	83	5	12
2	36	41	70	20	90	104	20	34
3	28	40	70	20	101	118	31	48
4	32	41	70	20	112	130	42	60
8	24	41	69	20	126	150	57	81
16	22	42	68	20	148	184	80	116

The differences between the mean gains of the groups of rats receiving the different thiamin supplements, the standard errors of the differences and 't' values are presented in table 3. All differences in mean gains are statistically significant. Further, it is apparent that the differences in mean gains can be just as accurately ascertained at the end of 3 weeks of supplement feeding as at 5 weeks. The savings in time and cost for a 3-week feeding period in comparison with a 5-week feeding period are obvious. Coward ('38) has pointed out that for her rat colony a 3-week feeding period was satisfactory for vitamin B_1 tests and has shown that a test carried out in 2 weeks was almost as accurate as one continued for 3 weeks ('36).

TABLE 2

The standard errors and 't' values of the mean gains in weight of six groups of rats after 3 weeks and after 5 weeks of supplemental feeding of synthetic crystalline thiamin

THIAMIN	NUMBER OF RATS	3 WEEKS			5 WEEKS		
		Mean gain	Standard error of mean	't' [1] value	Mean gain	Standard error of mean	't' value
µg.		*gm.*	*gm.*		*gm.*	*gm.*	
1	25	5	2.1	2.4	12	3.0	4.0
2	36	20	1.4	14.3	34	1.9	17.9
3	28	31	2.3	13.5	48	2.6	18.5
4	32	42	1.6	26.2	60	2.5	24.0
8	24	57	2.6	21.9	81	3.4	23.8
16	22	80	3.7	21.6	116	5.4	21.5

[1] For samples of *n* larger than 19, a 't' value greater than 2.1 indicates significance at the 5% probability level, and a 't' value greater than 2.9 indicates significance at the 1% probability level (Fisher, '36, table IV).

TABLE 3

The standard errors and 't' values of differences between the weight gains of groups of rats fed supplements of synthetic crystalline thiamin

INTERVAL OF THIAMIN SUPPLEMENT	3 WEEKS			5 WEEKS		
	Difference	Standard error	't' value	Difference	Standard error	't' value
	gm.	*gm.*		*gm.*	*gm.*	
1–2	15	2.4	6.2	22	3.3	6.7
2–3	11	2.5	4.4	14	3.1	4.5
3–4	11	2.7	4.1	12	3.6	3.3
4–8	15	2.9	5.2	21	4.1	5.1
8–16	23	4.4	5.2	35	6.2	5.6

In the assay of natural foods for thiamin by the biological method, the minimum number of experimental rats that must be used to obtain reliable results is also extremely important. It is apparent from the 't' values given in table 3 that a mean difference only half as large as that found would be statistically significant, other factors being maintained, or that the difference found would have been satisfactory if less than half the number of rats in each treatment had been used.

In order to obtain an accurate estimation of the error variance of gain in weight of individual rats at different periods after supplemental feeding of thiamin, twenty-one rats from each group or treatment were selected at random and the methods for the analysis of variance of an experiment having twenty-one replications and six treatments were applied, separate analyses being made for the 3-week and 5-week periods. The results are presented in table 4. From the total variance of the gain in weight of the 126 rats measured at each period the variances due to treatment and replication have been subtracted leaving variance or mean square deviation for experimental error. The square root of the error variance yields the standard error of a single observation; the standard errors are 12.4 gm. and 17.6 gm. for gain in weight per rat after 3 weeks and after 5 weeks respectively. If a difference of 11 gm. gain in weight between two treatments at 3 weeks is considered satisfactory, it is then a simple calculation to determine the number of rats required in each treatment to demonstrate the statistical

TABLE 4

Analysis of variance of gain in weight of twenty-one rats in each of six thiamin treatments at 3 weeks and at 5 weeks

SOURCE OF VARIANCE	DEGREES OF FREEDOM	3 WEEKS VARIANCE	5 WEEKS VARIANCE
Treatment	5	14,790	27,173
Replication	20	82	168
Error	100	155	311
Total	125	..	..
Standard error, gain in weight of a single rat		12.4	17.6

significance of this difference.[4] About twelve and fifteen rats, respectively, would be required to demonstrate the significance of differences of 11 gm. and of 14 gm. for the 3- and for the 5-week periods. From this standpoint the 3-week period is not only more economical in time and labor but also in number of rats required to yield statistically significant differences. Larger differences such as are found between 1- and 3- or between 2- and 4-µg. thiamin supplements can, of course, be proved with smaller numbers.

The curves of response for 3 weeks and for 5 weeks have been calculated according to the method of Coward ('38, fig. 2). The equation of the curve is $y = \bar{y} + b \log X$, where

y = gain in weight in grams during feeding period
$\bar{y}$ = mean value of y
X = daily dose of thiamin in micrograms
x = log of daily dose of thiamin in micrograms
$\bar{x}$ = mean value of x
b = regression coefficient, or $\frac{\Sigma y\,(x-\bar{x})}{\Sigma\,(x-\bar{x})^2}$

The actual mean gains in weight, especially in that portion of the curve in which the curvature is greatest, fall very close to the calculated curves for the gains in weight. Experiments which result in gains in weight falling in this portion of the curve are, therefore, the most sensitive.

The use of a curve of response in assaying foods of unknown vitamin content has been fully discussed by Coward ('38).

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[4] Calculation

Standard error of mean $\frac{12.4}{\sqrt{n}}$

Standard error of difference between two means $\frac{12.4 \times \sqrt{2}}{\sqrt{n}}$

't' value required for minimum level of significance when $n = 10$ more or less is approximately 2.2. $\frac{12.4 \times \sqrt{2} \times 2.2}{\sqrt{n}}$

Assuming that a minimum difference of 11 gm. is as great as may be conveniently proved. $\frac{12.4 \times \sqrt{2} \times 2.2}{\sqrt{n}} = 11$

Solution $n = 12$

SUMMARY AND CONCLUSIONS

1. To determine the growth response of rats to graded doses of thiamin, 192 rats were given a thiamin-free diet at 21 days of age; at depletion of thiamin stores after about 20 days, twenty-five rats were used as controls, and 167 rats were fed daily doses of synthetic crystalline thiamin at six levels (1, 2, 3, 4, 8 and 16 µg.) for a period of 5 weeks. The results show a definite curvilinear relationship between the mean gains in weight after 3 weeks and after 5 weeks and the quantity of thiamin administered; $y = 3.0 + 62.3 \log X$ for 3 weeks, and $y = 9.2 + 84.9 \log X$ for 5 weeks.
2. A statistical analysis of the results of a 5-week feeding period and those of a 3-week feeding period shows that the shorter period gives as significant results as the longer period.
3. The number of rats necessary for a significant biological assay of foods for their thiamin content for a particular colony has been determined to be twelve.
4. When supplements providing quantities of thiamin between 1 and 4 µg. are fed to groups of twelve rats six times a week, the responses should be sufficiently accurate to distinguish differences of 1 µg.
5. Curves of response relating the doses of thiamin to gains in weight are given for the 3-week and the 5-week feeding periods.

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LITERATURE CITED

COWARD, K. H. 1936 The influence of the length of the test period on the accuracy obtainable in a vitamin B_1 test. Biochem. J., vol. 30, p. 2012.

———— 1938 The biological standardization of the vitamins. William Wood and Co., Baltimore.

DANIEL, E. P., AND H. E. MUNSELL 1937 Vitamin contents of foods. U. S. Dept. Agric. Misc. Pub., no. 275.

Fisher, R. A. 1936 Statistical methods for research workers. Oliver and Boyd, London.

Heyroth, F. F. 1936 The use of various rat assay methods in comparing crystalline vitamin B_1 preparations. Biochem. J., vol. 30, p. 645.

Kinnersley, H. W., J. R. O'Brien and R. A. Peters 1935 Crystalline vitamin B_1. Biochem. J., vol. 29, p. 701.

Leong, P. C., and L. J. Harris 1936 Antineuritic potency of synthetic and natural crystalline vitamin B_1 as determined by the 'Bradycardia' method. Biochem. J., vol. 31, p. 672.

Waterman, R. E., and M. Ammerman 1935 Studies of crystalline vitamin B. V. The effect of graduated doses on growing rats. J. Nutrition, vol. 10, p. 35.

Williams, R. R. 1938 The chemistry of thiamin (vitamin B_1). J. Am. Med. Assoc., vol. 110, p. 727.

PRODUCTION OF GOITER IN RATS WITH RAW AND WITH TREATED SOY BEAN FLOUR

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Recent reviews indicate that endemic goiter can be controlled, except in certain areas of New Zealand, by administration of iodine. However, discrepancies in the data furnished by surveys leave considerable doubt that iodine deficiency per se is the primary cause of goiter. Numerous surveys have shown that iodine intake is low in goiter areas but is not lower than the intake in many goiter-free areas. The nature of endemic goiter has been reviewed recently by McCarrison ('37) and by Hellwig ('37). They both agree that there are two types of endemic goiter. In mountainous areas it occurs as a diffuse parenchymatous goiter in childhood and as adeno-parenchymatous nodular goiter in adults with its degenerative forms. The characteristic type in plains areas is a diffuse colloid goiter. A complete explanation of these differences probably depends on more than a simple iodine deficiency.

With the exception of studies on goiter in rabbits (Webster and Chesney, '30) most recent experiments have been made upon rats. The rachitogenic diet of Steenbock and Black or modifications of it, is the only diet reported which has consistently produced goiter in rats in different laboratories. Levine, Remington and von Kolnitz ('33) made a critical study of the goiter obtained with this diet. Hellwig ('35) was able to produce goiter in rats by feeding rolled oats, cornmeal, distilled water and calcium but the iodine content of the diet was not determined. Under certain experimental conditions McCarrison ('33) found that some native diets of India will produce goiter in rats. Diets of highly purified materials even though they are low in iodine have uniformly failed to produce enlarged thyroids in rats.

In order to study some of the dietary factors which may influence the thyroid gland, studies were started with a purified diet low in iodine (Carpenter and Sharpless, '37). With the rats used, satisfactory goiter was obtained neither with this diet nor with the diet of Levine, Remington and von Kolnitz ('33). The addition of substances which have been reported to increase the need for iodine (such as unsaturated fats, calcium, chlorides, fatty acids and various nitriles) did not produce a marked degree of enlargement. Since McCarrison ('34) had reported that soy beans would produce a goiter in rats which was not prevented by iodine, a diet composed mostly of soy bean flour was fed and a marked thyroid enlargement was obtained (Sharpless, '38).

Soy beans have a high nutritive value (Horvath, '38) and probably provide as complete a diet as can be obtained from any single vegetable source. The protein is adequate for promoting normal growth and is 'physiologically good.' The content of vitamins A, B complex and E is adequate and it has a high mineral content, especially of calcium and phosphorus.

EXPERIMENTAL

Rats, both male and female, weighing 60 to 80 gm.; were used for all of the studies. They were bred on an adequate stock ration which has been described elsewhere (Carpenter and Sharpless, '37). It was found that animals which weighed more than 60 gm. adjusted themselves to the diet more readily than smaller animals. Wire cages with wire mesh bottoms were used. They were washed frequently. These rats were kept in a room with only a few other experimental animals. Those on diets which had some iodine added were fed in a separate room. The soy bean flour was unprocessed and was prepared from Michigan-grown beans. Precautions were taken to insure fresh flour and avoid rancidity. Different samples obtained over a period of 4 years have varied from 15 to 20% in ether extractable material but have been uniformly satisfactory in producing goiter.

Formulae of the diets studied are shown in table 1. Rats fed the first diet (no. 62) which contained 93% soy bean flour did not do well. For the first few days they had diarrhea, were bloated and about 50% of them died. Those that survived developed a tolerance for the diet and grew to maturity although they never had a healthy appearance. When 5% of butterfat and 13% sugar displaced 18% soy bean flour (diet 62 A), all of the rats ate the diet and grew at approximately 40% of the normal rate. Because of the numerous reports on the goiterogenic effect of calcium, 3% of calcium carbonate was fed. In later experiments 0.5% of calcium carbonate was used (diet 153), and goiters of the same magnitude were obtained. Rats fed this diet grew at about 60% of the normal rate. With some exceptions they attained normal adult size, reproduced and in general appeared normal. However, if the diet was old or rancid, they did not grow so well and they lost much of their hair. Although the desirability of improvement was recognized this diet was considered satisfactory for further study.

TABLE 1

Formulae of soy-bean goiterogenic diets

		62	62 A	143	153	168	203	204	213
Unprocessed soy bean flour	%	93.0	75.0	75.0	75.0	75.0	75.0	75.0	35.0
Calcium carbonate	%	3.0	3.0	0.5	0.5	0.5	0.5	0.5	0.5
Sodium chloride	%	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Butterfat	%	0.0	5.0	5.0	5.0	5.0	5.0	0.0	5.0
Yeast	%	3.0	3.0	3.0	3.0	3.0	0.0	3.0	3.0
Sucrose	%	0.0	13.0	15.5	15.5	15.5	18.5	20.5	55.0
Viosterol—15 drops per kilogram food		—	—	—	—	+	—	—	—
Iodine as K I		0.0	0.0	5 mg. per kg.	0.0	0.0	0.0	0.0	0.0

In order to determine whether the supplements were of value, the yeast was omitted from diet 203 and the butterfat was omitted from diet 204. Goiters developed with both diets but growth was not so good as when both supplements were present. To be sure that a rachitic condition was not contributing to the development of goiter, viosterol was added to one diet (diet 168). Viosterol had no apparent effect on the growth or size of goiter. Iodine was added to one diet (143) to determine whether the goiterogenic property was active in the presence of iodine. Finally the soy bean flour content was reduced to 35% and sugar substituted (diet 213). There is less iodine in sugar than in soy bean flour and therefore this change effected a decrease in the iodine content of the diet. If there is a direct relationship between iodine deficiency and goiterogenic activity, this diet should be more goiterogenic than any of the others.

Iodine was determined in the dried thyroid glands by the method of Trevorrow and Fashena ('35). In the diet oxidation was carried out in an atmosphere of oxygen and the oxidation products were collected in a dichromate-sulfuric acid mixture. The determination was then completed by the Trevorrow and Fashena method. When freshly purified chemicals were used and care observed in each step of the procedure the method gave satisfactory results.

RESULTS

The degree of thyroid enlargement and the iodine content of the glands are shown in table 2. To avoid confusion only results on the normal, 75% soy bean flour, 35% soy bean flour and iodine containing soy bean flour diets are recorded. There was no significant difference between goiters obtained with the other diets and those shown for diet 153.

The average thyroid size of 130 rats fed the goiterogenic diet for 7 weeks was approximately four times normal. Some rats developed larger thyroids if fed the diet longer but the average of a number of rats fed for 160 days was about the same as that shown for 49 days. As an exception, a thyroid nine times normal size and weighing 230 mg. has been obtained in a rat fed for 9 months. A period shorter than 7 weeks has not been satisfactory. Seven rats in table 2 show that goiters were not well developed in 4 weeks. For these reasons all of the studies were based on an experimental period of 7 weeks.

Stock animals had a thyroid iodine content of 4.2 μg. which was 0.1% of the dry weight. This is within normal limits but it approaches the value at which histological changes might be expected. Therefore it will be called the minimum requirement in this report. The enlarged thyroids had an average total iodine content of 0.6 μg which was 0.005% of the dry weight. Thyroids of rats fed diet 153 for 4 weeks and diet 213 for 7 weeks had a similar iodine content but the glands were smaller. Rats fed diet 143 which contained added iodine had an average iodine content of 10.6 μg or 0.21% of the dry weight, showing that the thyroid of rats fed soy bean flour reacts in a normal manner to ingested iodine.

TABLE 2

Production of goiter with soy bean flour and prevention with iodine

DIET	IODINE CONTENT OF DIET	TIME ON EXPERIMENT	NUMBER OF RATS	AVERAGE THYROID WEIGHT		IODINE CONTENT	
				Total	Per 100 gm. Body weight	Average Total	Dry weight Basis
	μg/kg.	*Days*		*mg.*	*mg.*	*μg.*	*%*
Stock	95		31	20.3	10 ± 0.3	4.2	0.10
153	50	49	130	57.2	40 ± 0.8	0.6	0.005
153	50	29	7	28.2	25 ± 1.0	0.3	0.009
213 (35% soy bean flour)	35	49	22	23.0	24 ± 1.8	0.4	0.008
143 (0.5 mg.% K I)	3875	49	14	16.8	11 ± 0.3	10.6	0.21

HISTOLOGY

Microscopically the glands of stock animals were normal. The follicles were lined with low cuboidal cells and were well filled with colloid. The colloid did not stain so darkly as the colloid in glands getting more iodine. The goiters had follicles lined with high cuboidal or columnar cells showing hypertrophy and hyperplasia. In the larger goiters there was practically no colloid and the epithelium had folded into the follicles so that there was a great increase in the number of very small follicles. The first change appeared to be one of hypertrophy rather than hyperplasia. Iodine prevented this change and produced a normal gland.

It is shown that iodine prevented the goiter but it is not clearly shown that iodine deficiency alone produced the goiter. Diet no. 213 which contained 35% soy bean flour had a lower iodine content than diet 153. However, the goiters obtained with this diet were not so large as those obtained with diet 153. The difference in thyroid weight is statistically significant when glands of rats fed diet 213 are compared with the whole series fed diet 153 and also (not shown in table 2) when compared with the litter-mate controls fed diet 153. If this observation is correct, the iodine requirement of rats fed the goiter diet should be greater than normal, and there should be some means of changing the goiterogenic properties of the diet without changing its iodine content or nutritional properties. These studies were made and are shown below.

Iodine requirements of rats fed soy bean flour diet no. 153. Diet no. 153 (75% soy bean flour) was used as the basal diet and iodine in the form of potassium iodide was added. Litters were divided among all of the diets so that the stock diet is a direct control of all of the diets. Results and the diets studied are recorded in table 3.

Twenty micrograms and 40 μg. of iodine added to a kilogram of food caused a reduction in size from 36 to 15 mg. per 100 gm. body weight. As shown in the table, the glands are significantly larger than normal. One hundred micrograms of added iodine (diet 209) caused a reduction in size to 12 mg. per 100 gm. body weight which is also significantly larger than normal. Five hundred micrograms (diet 210) produced glands that weighed 11 mg. per 100 gm. of body weight which is not significantly larger than normal.

All of the diets except 210 produced glands with less than 0.1% iodine and therefore furnished less than the minimum requirement. The amount of iodine necessary to produce a gland with an iodine content of 0.1% was not determined exactly but from the figures shown it can be estimated that 50 µg. added to a kilogram of diet no. 209 would furnish approximately the minimum requirement. Which means that an iodine content of 200 µg per kilogram or approximately 50 µg per 1000 calories is the minimum amount of iodine required to produce a normal gland in rats fed the soy bean flour diet. With the figures shown it can be calculated that the amount of iodine necessary to produce an optimum iodine concentration in the thyroid (around 0.2% of the dry weight, Remington, Remington and Welch, '37) is not less than 200 µg. per 1000 calories. In a normal diet, the optimum requirement is around 80 µg. per 1000 calories.

TABLE 3

Iodine requirement of rats fed goiterogenic diet

DIET	IODINE CONTENT OF DIET	NUMBER OF RATS	AVERAGE THYROID WEIGHT		IODINE IN THYROID		SIGNIFICANCE RATIO TO STOCK ANIMALS	PROBABILITY
			Total	Per 100 gm. Body weight	Average Total	Dry weight		
	µg/kg.		*mg.*	*mg.*	*µg*	%		%
Stock	95	31	20.3	10 ± 0.3	4.17	0.101		
153	50	21	47.4	36 ± 1.4	0.48	0.006	7.4	0
223	70	6	17.0	15 ± 0.6	0.57	0.015	4.4	0
208	90	50	17.7	15 ± 0.4	1.10	0.021	6.5	0
209	150	19	13.3	12 ± 0.4	2.3	0.075	2.5	1.2
210	550	10	15.7	11 ± 0.7	6.9	0.162	0.9	39

Our stock diet contained around 25 µg of iodine per 1000 calories and produced glands very similar in size and iodine content to those reported by Levine, Remington and von Kolnitz ('33 a) in stock rats fed a similar amount of iodine. Rats fed the rachitogenic goiter diet also require a similar amount. The soy bean flour diet however requires twice as much iodine as the normal. The question arises: Is this effect produced by some property of the soy beans? Raw soy bean flour has a flavor that is objectionable to many animals and it is not so nutritious raw as cooked (Horvath, '38). In order to obtain additional information on the nature of the goiterogenic property, the effect of heat and of fat solvents on the goiterogenic ability of soy bean flour was studied.

Effect of heat and of fat solvents on goiterogenic action of soy bean flour. The diets used in these experiments were all modifications of diet 153. Litter-mate controls were fed diet 153 in each study. In place of unprocessed soy bean flour in diet 153, soy bean flour treated by one of the following three methods was used: 1) extracted with ether or acetone; 2) autoclaved for 30 minutes at 20 pounds pressure; 3) steam generated from iodine-free water was passed through a 10-inch column of the flour for 30 minutes. The methods of treatment did not change the iodine content but fat extraction increased the iodine content per 1000 calories. Results of these experiments are shown in table 4.

TABLE 4

Effect of treatment on goiterogenic property of soy bean flour

DIET	NUMBER OF RATS	AVERAGE THYROID WEIGHT		IODENE CONTENT		SIGNIFICANCE RATIO	PROBABILITY
		Total	Per 100 gm. Body weight	Average Total	Dry weight		
		mg.	*mg.*	*µg*	%		%
169 ether extracted flour[1] 225 acetone extracted flour	19	28.5	21 ± 0.99	1.38	0.025	5.04	0
153 littermate controls	14	52.8	39 ± 2.19	0.95	0.009		
184 autoclaved flour	24	53.8	28 ± 1.2	0.63	0.006	2.48	1.2
153 littermate controls	21	62.9	39 ± 2.7	0.35	0.003		
150 steamed flour	20	31.3	19 ± 0.99	0.79	0.009	4.6	0
153 littermate controls	20	47.7	36 ± 2.3	0.51	0.004		

[1] Results with these two diets were identical and therefore they are recorded together.

All three methods of treating the soy bean flour caused a significant reduction in its goiterogenic activity. Ether or acetone extracted flour caused a reduction in thyroid weight from 39 to 21 mg. per 100 gm. of body weight. The total iodine content of the glands was slightly higher than that of the controls and the per cent content was much higher. Since some increase in iodine content might be expected because the iodine content of the diet per 1000 calories was slightly higher, the higher iodine content of the gland can only be interpreted in the light of results with the other diets. Autoclaved flour reduced the average thyroid size from 39 to 28 mg. per 100 gm. body weight. This is not so significant a decrease but there is only a probability of one in a hundred that it occurs by pure chance. The average iodine content per gland was raised from 0.35 to 0.63 μg. Steamed flour caused a reduction from 36 to 19 mg. per 100 gm. body weight and also an increase in the average iodine content.

Microscopically, the glands of animals fed the treated flours showed less hyperplasia than their controls. There was considerable hypertrophy of the cells and very little colloid present.

DISCUSSION

The results show that the goiterogenic action of the diet is not wholly dependent upon its iodine content. However, the glands were larger than normal in all of the experiments. The thyroids of rats fed the treated flours were similar in size to those fed the modified rachitogenic goiter diet (Levine, Remington and von Kolnitz, '33) which contained the same amount of iodine. It is therefore possible that the iodine content is low enough to produce some thyroid enlargement.

The experiments show that the high fat content of the diet does not produce the goiter. Extraction of fat decreases the enlargement but steaming the flour has the same effect even though the fat is not extracted.

Crotti ('38) has recently reported that a fungus will produce goiter in rabbits, guinea pigs and dogs. The fungus is present in goiterogenic cabbage, water supplies in endemic goiter areas and in fresh goiter material. Steam had no effect on the fungus. Since steam significantly reduced the goiterogenic effect of soy bean flour, it is improbable that the goiterogenic agent in soy bean flour is this fungus. Crotti also found that small amounts of iodine had little effect on the goiterogenic action of the fungus whereas small amounts of iodine prevented the goiter produced by soy bean flour.

In view of the intimate relation of the thyroid and pituitary, it is always possible that thyroid changes may be secondary to changes in the pituitary. Pituitaries were weighed in all experiments and no definite change in weight could be detected in the 7-week experimental period. Longer experimental periods produced slightly enlarged pituitaries.

Marine, Baumann, Spence and Cipra ('32) have shown that organic cyanides stimulate the thyroid by depressing the oxidative systems of the body. In preliminary studies, methyl nitrile did not produce goiter in rats even though the iodine intake was low. McCarrison ('33) also found that methyl nitrile did not produce goiter in rats even in doses far greater than the amount that could be obtained from their food. However, soy beans are relatively rich in cyanogen and yield considerable quantities of cyanide on enzyme hydrolysis. Without further study this must be considered as a possible goiterogenic agent in soy bean flour.

The functional state of the thyroid of rats fed the soy bean diet as shown by metabolism tests has been the subject of preliminary studies. While the data are too few to draw definite conclusions, there is no indication that the basal metabolic rate is lower than that of litter-mate controls of the same body weight. The basal rate is consistently a little higher but probably not more than the experimental error expected with the method.

SUMMARY

1. A diet composed of unprocessed soy bean flour, yeast, sugar, butterfat, sodium chloride and calcium carbonate, when fed to rats, produces thyroid enlargement of four times normal in an experimental period of 7 weeks.

2. The minimum iodine requirement of rats fed the goiterogenic diet is two times normal or approximately 50 μg. per 100 calories of food. The optimum iodine requirement is approximately 200 μg per 1000 calories which is also twice normal.

3. Unprocessed soy bean flour contains a positive goiterogenic property that is removed or destroyed by fat solvents (ether or acetone) or by steam.

LITERATURE CITED

CARPENTER, MARY DORIS, AND GEORGE R. SHARPLESS 1937 A study of the effect of vitamin B and iodine on the weight, iodine content and structure of the thyroid gland of the rat. J. Nutrition, vol. 13, pp. 235–247.

CROTTI, ANDRE 1938 Research on the etiology of endemic goiter. Trans. Internat. Coll. Surgeons, vol. 1, p. 38.

HELLWIG, C. A. 1935 Experimental goiter. Functional, chemical and histological studies. Arch. Path., vol. 19, pp. 364–371.

——— 1937 The diffuse colloid goiter. Surg. Gyn. and Obst., vol. 64, pp. 604–608.

HORVATH, A. A. 1938 The nutritional value of soy beans. Am. J. Dig. Dis., vol. 5, pp. 177–183.

LEVINE, HAROLD, ROE E. REMINGTON AND HARRY VON KOLNITZ 1933 Studies on the relation of diet to goiter. I. A dietary technic for the study of goiter in the rat. J. Nutrition, vol. 6, pp. 325–345.

——— 1933 a Studies on the relation of diet to goiter. II. The iodine requirement of the rat. Ibid., vol. 6, pp. 347–354.

McCARRISON, ROBERT 1933 Food and goiter. Brit. Med. J., vol. 2, pp. 671–675.

——— 1934 The goiterogenic action of soya-bean and ground-nut. Ind. J. Med. Res., vol. 21, pp. 179–181.

——— 1937 The problem of endemic goiter. Brit. Med. J., vol. 1, pp. 29–31.

MARINE, D., E. J. BAUMANN, ALLAN M. SPENCE AND ANNA CIPRA 1932 Further studies on the etiology of goiter with particular reference to the action of cyanides. Proc. Soc. Exp. Biol. and Med., vol. 29, p. 772.

REMINGTON, ROE E. 1937 Improved growth on iodine deficient diets. J. Nutrition, vol. 13, pp. 223–233.

REMINGTON, ROE E., JOHN W. REMINGTON AND SARAH S. WELCH 1937 The thyroid gland of the normal rat; size, dry matter and iodine content. Anat. Rec., vol. 67, pp. 367–376.

SHARPLESS, GEORGE R. 1938 A new goiter producing diet for the rat. Proc. Soc. Exp. Biol. and Med., vol. 38, pp. 166–168.

TREVORROW, VIRGINIA, AND GLADYS J. FASHENA 1935 The determination of iodine in biological material. J. Biol. Chem., vol. 110, pp. 29–38.

WEBSTER, BRUCE, AND ALAN M. CHESNEY 1930 Studies in the etiology of simple goiter. Am. J. Path., vol. 6, pp. 275–284.

THE UTILIZATION OF THE CALCIUM IN VARIOUS GREENS[1]

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The importance of the green leafy vegetables as sources of dietary calcium has been responsible for many investigations of the availability of their calcium. The calcium of kale (Fincke and Sherman, '35) and of Chinese cabbage (Kao, Conner and Sherman, '38) was found to be nearly as well utilized by rats as that of milk. Mallon, Johnson and Darby ('33) have reported the calcium of lettuce as more efficiently used by young women than that of milk. The maintenance requirements of young women were found by Blatherwick and Long ('22) to be satisfied by the calcium of lettuce, spinach or cabbage. Fincke and Garrison ('36) have reported that the calcium of kale is considerably more available to young women than that of spinach.

Others have found the calcium of spinach poorly utilized both by dogs (McClugage and Mendel, '18) and by young rats (Fincke and Sherman, '35; Fairbanks and Mitchell, '38; Tisdall and Drake, '38). The low availability of the calcium of spinach has been attributed to its oxalate content. Sherman and Hawley ('22) have reported that children utilize the calcium of milk better than that of spinach. That spinach tends to produce a negative calcium balance when introduced into the diet of infants has been shown by Edelstein ('32). This decrease in calcium retention of infants fed spinach was not due to its fiber content (Schlutz, Morse and Oldham, '33) since cellulose, in the form of Celluflour, had no effect on the calcium balance. Likewise for rats Bloom ('30) has reported that though dried spinach in the diet caused less calcium storage than the ash of the same amount of spinach, the fiber content was not responsible for the decreased retention since filter paper fed with the ash had no such effect.

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EXPERIMENTAL

In these experiments the procedure used was essentially that developed by Fincke and Sherman ('35) in their work on the availability of calcium in spinach and kale. It was planned to determine the utilization of the calcium of turnip greens, tendergreen, collards and New Zealand spinach, as well as of kale grown under different conditions.

The experimental animals were albino rats reared by mothers on a stock diet of one-third whole milk powder and two-thirds ground whole wheat, which is known to support normal growth and calcification. The experimental diets were patterned after the stock diet, skim milk powder and filtered butter fat being used in place of the whole milk powder. In the control diet practically all of the calcium was supplied by the skim milk; in the diets containing the greens one-half of the skim milk was replaced by enough of the green to supply an equal amount of calcium, the difference in weight being made up with the cornstarch. The animals were fed the experimental diets from 28 days to 60 days of age, a period during which the rate of growth and calcification is very rapid. The utilization of the calcium in these diets was to be measured for this period.

The greens were grown under similar conditions and harvested at approximately the same degree of maturity. After being thoroughly washed, they were dried at 60° to 65°C. and ground in a ball mill until fine enough to pass through a 40-mesh sieve. A homogeneous sample of each sufficient for the entire experimental period was prepared.

Calcium was determined gravimetrically by the modification of the McCrudden method developed by Sherman and MacLeod

('25), and phosphorus by the method of the Association of Official Agricultural Chemists. The method of Arbenz ('17) was used to determine oxalic acid.

The same lot of both skim milk powder and whole wheat was used throughout the experimental period, the respective calcium contents equalling 1.40% and 0.037%. The greens contained the following percentages of calcium: New Zealand spinach 0.03%, kale 2.30%, collards 2.33%, turnip greens 2.39%, tendergreen 3.07%. The only one of the greens found to contain oxalate was New Zealand spinach with a content of 4.8% of anhydrous oxalic acid.

TABLE 1

Composition of the diets and their calcium and phosphorus contents

	CONTROL	KALE	NEW ZEALAND SPINACH	COLLARD	TURNIP GREEN	TENDER-GREEN
Whole wheat	65	65	65	65	65	65
Butterfat	10	10	10	10	10	10
Skim milk powder	22	11	11	11	11	11
Cornstarch	3	7	—	7	8	9
Greens	—	7	14	7	6	5
NaCl	1.3	1.3	1.3	1.3	1.3	1.3
Calcium—%	0.327	0.339	0.320	0.338	0.314	0.325
Phosphorus—%	0.418	0.357	0.366	0.314	0.360	0.351

The composition and analysis of the experimental diets are shown in table 1. The calcium contents of the diets were, as was planned, practically the same; the phosphorus contents were not widely different.

On being weaned at 28 days of age, the experimental animals were placed in individual cages with food and water provided ad libitum. A careful record of food consumption was kept so that the calcium intake of each animal would be known. The distribution of the animals to the various diets was made as even as possible in respect to initial weights and litter mates. At 60 days of age the animals were killed with chloroform. The gastrointestinal tract was removed, the weight of its contents determined and then discarded. The weight of the rat minus the weight of the gastrointestinal contents has been termed the net body weight. The body was charred over a low flame and then ashed in a muffle furnace at dull red heat. The ash was dissolved to dilute hydrochloric acid and made up to a suitable volume, aliquots of which were used for the calcium analyses.

Representative animals were killed at 28 days of age, and the percentage of calcium in their bodies determined by analysis. The average percentages of calcium found in ten males and eleven females of this age were 0.66 ± 0.04% and 0.69 ± 0.03%, respectively (on the basis of live weight). These values were used to calculate the calcium present in the 28-day-old rats at the time they were placed on the experimental diets.

RESULTS AND DISCUSSION

The growth and food consumption of the animals during the experimental period are shown in table 2. Growth was greatest on the control diet and smallest on the New Zealand spinach diet. On the other diets containing greens, growth was about

TABLE 2

Growth and food consumption on the various diets

DIET	NUMBER OF RATS	INITIAL WEIGHT AT 28 DAYS	TOTAL GAIN IN WEIGHT	NET WEIGHT AT 60 DAYS	FOOD EATEN	GAIN PER GRAM OF FOOD EATEN
			Males			
		gm.	*gm.*	*gm.*	*gm.*	
Control	4	55	85	130	293	0.290
Turnip green	6	54	64	108	252	0.254
Tendergreen	6	53	66	109	258	0.256
Collard	6	56	56	103	249	0.225
Kale	3	55	61	108	255	0.240
New Zealand spinach	.5	53	18	60	149	0.121
			Females			
Control	5	50	62	103	249	0.249
Turnip green	4	46	56	95	240	0.233
Tendergreen	4	48	51	91	233	0.215
Collard	3	46	46	85	229	0.201
Kale	6	50	42	84	235	0.174
New Zealand spinach	3	52	16	55	152	0.105

equal to, or slightly less than, that on the control diet. Besides gaining but little weight, the animals on the New Zealand spinach diet were weak and unkempt in contrast to the normal and healthy appearance of the animals on the other diets. Smaller food consumption does not entirely account for the poor growth on the New Zealand spinach diet, for the gain in weight per gram of this diet eaten was only about one-half that of the other diets.

The calcium contents of the rats at the end of the experimental period are shown in table 3 expressed both as total and as percentage of net weight. Neither of these values in themselves measures calcium utilization accurately, for they do not take into account differences due to variations in initial store or food consumption. A more exact value for measuring utilization is expressed in the calcium utilization factor, which is the ratio of the calcium retained to that ingested. The difference between the calcium found by analysis at the end of the experimental period and that calculated to have been present at 28 days equals the calcium retained.

The calcium utilization factor of the control diet was the greatest, showing the ready availability of the milk calcium, while that of the turnip green diet was about equal to that of the control diet. A much larger number of animals would be necessary to determine whether this small difference was significant. From the calcium utilization factors of the tendergreen, collard and kale diets it appears that the calcium of these greens was not utilized quite as well as that of the turnip greens and the milk. The calcium utilization factor of the New Zealand spinach diet was extremely small compared with that of the other diets, equalling only about one-quarter that of the control diet. Apparently, not only was the calcium of the New Zealand spinach unavailable, but also the availability of the calcium of the milk in the diet was reduced.

Although New Zealand spinach (Tetragonia expansa) and spinach (Spinacea oralacea) are not of the same family they resemble one another in respect to their relatively high content of oxalate. Several groups of workers (Fincke and Sherman,

TABLE 3

Calcium content of 60-day-old rats on the various diets

DIET	NUMBER OF RATS	Ca IN BODY AT 60 DAYS		Ca IN BODY AT 28 DAYS (CALCULATED)	Ca RETAINED (CALCULATED)	Ca IN FOOD EATEN	Ca UTILIZATION FACTOR
		Total	Per cent of net weight				
		Males					
		gm.	%	*gm.*	*gm.*	*gm.*	
Control	4	1.129 ± 0.134[1]	0.87 ± 0.02[1]	0.361 ± 0.063[1]	0.767 ± 0.079[1]	0.967 ± 0.128[1]	0.80 ± 0.05[1]
Turnip green	6	0.978 ± 0.086	0.91 ± 0.04	0.358 ± 0.045	0.620 ± 0.041	0.808 ± 0.081	0.77 ± 0.03
Tendergreen	6	0.982 ± 0.076	0.90 ± 0.01	0.352 ± 0.047	0.630 ± 0.035	0.850 ± 0.040	0.74 ± 0.03
Collard	6	0.954 ± 0.101	0.93 ± 0.03	0.369 ± 0.036	0.585 ± 0.076	0.846 ± 0.065	0.69 ± 0.05
Kale	3	0.977 ± 0.039	0.91 ± 0.03	0.365 ± 0.038	0.612 ± 0.035	0.866 ± 0.063	0.71 ± 0.01
New Zealand spinach	5	0.410 ± 0.076	0.69 ± 0.05	0.341 ± 0.062	0.069 ± 0.041	0.478 ± 0.029	0.14 ± 0.08
		Females					
Control	5	0.985 ± 0.052	0.96 ± 0.03	0.342 ± 0.024	0.679 ± 0.038	0.820 ± 0.060	0.78 ± 0.06
Turnip green	4	0.891 ± 0.051	0.94 ± 0.04	0.319 ± 0.047	0.572 ± 0.018	0.769 ± 0.027	0.75 ± 0.04
Tendergreen	4	0.880 ± 0.070	0.97 ± 0.01	0.333 ± 0.054	0.547 ± 0.046	0.767 ± 0.049	0.71 ± 0.03
Collard	3	0.861 ± 0.016	1.01 ± 0.02	0.320 ± 0.012	0.541 ± 0.006	0.779 ± 0.018	0.70 ± 0.02
Kale	6	0.870 ± 0.058	1.05 ± 0.10	0.345 ± 0.035	0.525 ± 0.027	0.800 ± 0.052	0.66 ± 0.04
New Zealand spinach	3	0.459 ± 0.004	0.84 ± 0.03	0.356 ± 0.054	0.102 ± 0.050	0.486 ± 0.021	0.21 ± 0.10

[1] Average deviation of the mean.

'35; Fairbanks and Mitchell '38; and Tisdall and Drake, '38) have shown that the calcium of spinach is utilized poorly, if at all, by the growing rat and have attributed this to the oxalate content of spinach. Furthermore, Fairbanks and Mitchell ('38) found the calcium of calcium oxalate unutilizabe. About 75% of the calcium in the New Zealand spinach was probably in this insoluble form, for successive extractions of the New Zealand spinach with 2N HAc and 2N HCl dissolved only 25% of its calcium. It is of interest that this is about the same percentage of soluble calcium as that of 30% found by Horwitt, Cowgill and Mendel ('36) to be soluble on in vitro digestion of dried spinach with a pepsin-HCl mixture. Even if all the calcium in the New Zealand spinach diet were in the form of the oxalate, about 50% more oxalate would remain for possible combination with the other calcium in the diet. Mackenzie and McCollum ('37) have shown that potassium oxalate can decrease the utilization of calcium on borderline calcium diets, but not on high calcium diets. Consideration of our results in the light of this work reveals the possibility that the available calcium content of the diet was so lowered with 75% or more of the calcium in the New Zealand spinach bound in the form of unavailable calcium oxalate that the deleterious effect of the remaining oxalate was magnified, resulting in the decreased utilization of the calcium of the milk in the diet.

SUMMARY

In these experiments measurements were made of the utilization of the calcium of various dried greens in diets in which one-half of the calcium was supplied by the green and the other half by milk. These diets were fed to young rats from their twenty-eighth to sixtieth day of age, at which time they were killed and analyzed for calcium. The calcium utilization factor, the ratio of the calcium retained to that ingested, was used as a measure of the utilization of the calcium in the various diets during this period of rapid growth and calcification.

The results showed that the calcium of turnip greens was about as available as that of milk, while that of tendergreen, collards and kale was slightly less available. Not only was the calcium of New Zealand spinach utilized poorly, if at all, but also the utilization of the calcium of the milk in the diet was diminished by the presence of the New Zealand spinach.

LITERATURE CITED

ARBENZ, E. 1917 The quantitative determination of oxalic acid in foods and condiments. Mitt. Lebensm. Hyg., vol. 8, p. 98, cf. C. A. vol. 11, p. 2374.

BLATHERWICK, N. R., AND M. L. LONG 1922 The utilization of calcium and phosphorus of vegetables by man. J. Biol. Chem., vol. 52, p. 125.

BLOOM, M. A. 1930 The effect of crude fiber on calcium and phosphorus retention. J. Biol. Chem., vol. 89, p. 221.

EDELSTEIN, E. 1932 Der Einfluss von Gemüsezulagen auf den Stickstoff- und Mineralstoffwechsel des Kindes. Ztschr. f. Kinderheilk, Bd. 52, S. 483.

FAIRBANKS, B. W., AND H. H. MITCHELL 1938 The availability of calcium in spinach, in skim milk powder and in calcium oxalate. J. Nutrition, vol. 16, p. 79.

FINCKE, M. L., AND E. A. GARRISON 1936 The availability of the calcium of spinach and kale. Proc. J. Home. Econ., vol. 28, p. 572.

FINCKE, M. L., AND H. C. SHERMAN 1935 The availability of calcium from some typical foods. J. Biol. Chem., vol. 110, p. 421.

HORWITT, M. K., G. R. COWGILL AND L. B. MENDEL 1936 The availability of the proteins and inorganic salts of the green leaf. J. Nutrition, vol. 12, p. 237.

KAO, H., R. T. CONNER AND H. C. SHERMAN 1938 The availability of calcium from Chinese cabbage (Brassica Pekinensis, Rupr.). J. Biol. Chem., vol. 123, p. 221.

MACKENZIE, C. G., AND E. V. MCCOLLUM 1937 Some effects of dietary oxalate on the rat. Am. J. Hyg., vol. 25, p. 1.

MALLON, M. G., L. M. JOHNSON AND C. R. DARBY 1933 The calcium retention on a diet containing leaf lettuce. J. Nutrition, vol. 6, p. 303.

MCCLUGAGE, H. B., AND L. B. MENDEL 1918 Experiments on the utilization of nitrogen, calcium, and magnesium in diets containing carrots and spinach. J. Biol. Chem., vol. 35, p. 353.

SCHLUTZ, F. W., M. MORSE AND H. OLDHAM 1933 Vegetable feeding in the young infant. Influence on gastro-intestinal motility and mineral retention. Am. J. Dis. Child., vol. 46, p. 757.

SHERMAN, H. C., AND E. HAWLEY 1922 Calcium and phosphorus metabolism in childhood. J. Biol. Chem., vol 53, p. 375.

SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth and food. J. Biol. Chem., vol. 64, p. 429.

TISDALL, F. F., AND T. G. H. DRAKE 1938 The utilization of calcium. J. Nutrition, vol. 16, p. 613.

THE GROWTH, ACTIVITY, AND COMPOSITION OF RATS FED DIETS BALANCED AND UNBALANCED WITH RESPECT TO PROTEIN[1]

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INTRODUCTION

A balanced ration is one containing all the necessary nutrients in the proper proportions to promote the maximum bodily performance for which the ration is balanced and one which does not impair any bodily function or injure any internal organ. A ration balanced for growth, therefore, is one which will enable the animal to grow at a maximum rate. While it seems logical to suppose that the better balanced of two rations, for any particular purpose, will be more efficiently utilized as a whole than the less well balanced one, this has not been definitely proved. Also a ration balanced with respect to some particular nutrient may not be the one in which that nutrient is utilized to the greatest possible extent, but the bulk of all nutrients present, possibly best represented by the metabolizable energy content of the ration, probably will be more efficiently utilized than any other combination of nutrients (amounts consumed being the same) which will promote the same retention of the particular nutrient under consideration. For example, in a growing animal it is probable that protein retention will increase as the percentage of protein in the ration increases up to 18 or 20%, but this increase in retention will take place at a decreasing rate of efficiency of utilization; however, the efficiency with which the dietary energy is utilized by the animal would probably increase as the ration became better balanced.

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[1] The experimental data reported here have been taken from a thesis submitted in 1937 to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy in animal husbandry. Preliminary reports of this investigation have appeared previously (Hamilton, '35, '37).

The results to be reported here, and in the following paper, were obtained during a study started in July, 1934, concerned with the utilization of the energy of diets balanced and unbalanced with respect to protein for growing rats. The data to be reported in this paper deal with the effects of the percentage of protein in the diet upon the growth, activity, composition of gains, appetite and size of liver and kidneys of young growing male rats. The following paper will be concerned with the effects of the percentage of protein in the diet upon the utilization of its energy, with particular emphasis on the heat increments.

REVIEW OF PREVIOUS EXPERIMENTS

In addition to many observations based on the ad libitum method of feeding, that the growth-promoting values of diets increase as the percentage of protein in the diet increases up to 18 or 20, a few experiments in which a controlled method of feeding was used have demonstrated conclusively that this is so. Hogan and Pilcher ('33), using diets varying in protein content from about 7 to 33%, found that rats on the higher protein diets made better gains than did the rats on the lower protein diets, even though both diets furnished the same amounts of metabolizable energy. Hogan, Johnson and Ashworth ('35) and Johnson, Hogan and Ashworth ('36) compared a 10.0 with a 26.2% protein diet and showed that the rats on the higher protein diet made greater gains and stored more water, protein and energy, but less fat, than did their pair mates on the lower protein diet.

Forbes, Swift, Black and Kahlenberg ('35 a, '35 b) found on feeding rats equicaloric amounts of diets containing, respectively, 10, 15, 20 and 25% of protein, that with increased protein, up to at least 20%, there was an increase in total gains, an increase in protein stored and a decrease in fat gained per unit of protein gained. In a preliminary report, Mitchell and

Hamilton ('36) have also shown, with rats and with pigs, that the better balanced a ration is with respect to protein (so long as the percentage does not exceed the minimum required for maximum rate of growth), the greater is its growth-promoting value, and the gains of the higher protein-fed animal contained more protein and less fat per unit of protein gained than did those of comparable animals fed equicaloric amounts of a lower protein ration.

Forbes, Voris, Bratzler and Wainio ('38) fed quadruplets of rats diets containing 25, 30, 35 and 45% protein and found that equicaloric amounts of these diets produced decreasing rates of gains as the percentage of protein increased above 25. Mitchell and Smuts ('32) have also shown by the paired-feeding technic that the minimum percentage of meat protein capable of supporting maximum growth rate in rats was between 18 and 20%.

Concerning the effect of the percentage of protein in the diet upon the voluntary activity of rats, Slonaker ('31) concluded from ad libitum feeding tests that diets containing between 14 and 18% protein were most conducive to maximal spontaneous running. Forbes, Swift, Black and Kahlenberg ('35 a) concluded that the greater growth-promoting abilities of diets better balanced with respect to protein was due to a decreased activity of the rats fed the better balanced diets. This conclusion was made without any attempt to measure the activities of the rats, however.

As to the effects of the percentage of protein in the diet upon its palatability or upon the appetite of the animal, it is assumed all too frequently that the appetites of the experimental animals are infallible indices of the nutritive completeness of the diets fed. Fortunately for the very large number of ad libitum feeding experiments, this assumption seems to be generally true, but unfortunately this is not always the case. Beadles, Braman and Mitchell ('30) and Mitchell and Beadles ('30) demonstrated that the growth-promoting value of both cooked and uncooked garden peas could be improved by the addition of small amounts of cystine. However, in the cooked pea experiment there were fifty-nine food refusals by the rats on the deficient diet and eighty by the rats receiving the same amount of the supplemented diet. In the raw pea test, there were but twenty-one food refusals by rats receiving the deficient diet and forty-seven by the rats on the supplemented diet. Mitchell and Smuts ('32) also reported two cases, one with a meat protein diet and another with a corn protein diet, in which the rats receiving the diets with the lower growth-promoting value apparently had the better appetites.

PLAN OF INVESTIGATION

The general plan of this investigation consisted of a series of experiments on white rats involving paired-feeding, carcass analyses and metabolism studies. The experimental diets were complete and alike in all respects except for the percentage of protein which was varied from 4 to 54. The protein was supplied in the form of dried whole egg. Increases in the percentages of protein were accomplished at the expense of corn starch. The ingredients of the diets were as follows:

Constant factors	
Salts[2]	5.00%
Agar agar	1.00%
Cod liver oil[3]	3.00%
Filtered butterfat	8.00%
Variable factors	
Sucrose (This percentage was constant in all diets except the 54% protein diet, in which it was 2.35%)	10.00%
Purified corn oil[4] (Varied slightly so as to make a total of 19.4% fat in all diets)	8.00%
Cornstarch[5]	59.13% to 0%
Extracted whole dried egg[6]	5.87% to 69.80%

[2] Wesson ('32) slightly modified so as to contain traces of Co, Zn and Mn.

[3] E. L. Patch Co., Boston, Mass.

[4] Mazola.

[5] Contains no more than traces of nitrogen. A. E. Staley Mfg. Co., Decatur, Ill.

[6] Phenix-Kraft Cheese Co., Chicago, Ill. Extracted with ether until less than 1% fat remained.

The B vitamins were supplied in the form of Harris yeast concentrate, each rat receiving 0.5 mg. daily per gram of body weight. This amount of yeast concentrate was selected after preliminary paired-feeding experiments, using both high and low protein diets containing 20% fat, indicated that the above stated amount was ample for maximum growth rate.

The synthetic diets studied contained the following approximate percentages of protein (T.N. × 6.25): 4, 8, 12, 16, 18, 20, 22, 30, 42 and 54. All tests were of a comparative nature; the following comparisons having been made: 4 versus 8 (two tests), 8 versus 12 (two tests), 12 versus 16 (two tests), 8 versus 18, 16 versus 20, 18 versus 22, 22 versus 42, 20 versus 54 (two tests), and 20 versus 54 (cooked egg). The diets were compared in the following respect: 1) ability to produce gains in weight and in body length; 2) ability to produce retentions of energy and of nitrogen; 3) effects upon the appetite; 4) effects upon the voluntary activity of rats; 5) their contents of digestible nitrogen and metabolizable energy, and 6) in a few cases, their effects upon the weights of livers and kidneys in rats.

The comparative growth-promoting abilities of the various diets were determined by a series of fourteen 5- to 24-week paired-feeding tests. In each test, eight pairs of brother rats were used. Inbred white rats, approximately 4 weeks of age and weighing about 50 gm. at the start of the test, were paired as to litter, age, sex and weight. With few exceptions, notably the tests in which low-protein diets were fed, all tests continued until the rats had gained between 150 and 175 gm. At the termination of each test, all rats were etherized, their nose-to-anus lengths and empty body weights determined. Eventually all carcasses were analyzed for their total nitrogen and gross energy contents. While body weight and length increases were used as indications of the comparative growth-promoting abilities of the diet, the amounts of protein stored were used as the final criterion.

The rats on paired-feeding tests were kept in small four-compartment wire cages each compartment measuring 2.5 ×

5 (or 7) × 7 inches. These small cages were used in order to restrict the activity of the rats as much as seemed practicable and it was hoped also that the activity of pair-mates would be more nearly equal. All rats were kept in an air-conditioned room, the temperature of which was maintained at 28 ± 1°C. and the humidity at 43 ± 5%. The only work not done in the air-conditioned room was that in which the revolving cages were used in order to compare the voluntary activity of paired rats receiving the different diets.

In three paired-feeding tests, the usual paired-feeding technic was followed, i.e., pair-mates were fed the same amounts of food daily. In all other tests, a modified procedure was employed. This modified procedure consisted in the daily feeding of such amounts of the diets being compared that equal daily gains in weight resulted. This latter procedure, while furnishing quantitative data relative to the growth-promoting abilities of the diets used, had the added advantage that the maintenance requirements of pair-mates would be very nearly the same throughout the test. The digestibility of the protein and the metabolizable energy content of each diet were determined by the usual method. In most cases these values were determined after the rats had been on the paired-feeding test for at least 3 weeks. During the 14-day collection period, the rats were kept in glass-bottomed metabolism cages to enable accurate urine collections. Gross energy determinations were made on the urines and feces after drying at low temperatures.

The voluntary or running activity of three series of rats was determined by keeping, for several weeks, each series of eight pairs of rats in a battery of sixteen identical revolving cages (compare Mitchell, '32–'33) each of which was equipped with a revolution counter.

In the following presentation and discussion of results, each datum represents the average of at least eight values obtained on as many individual rats, unless otherwise made clear to the contrary. All data were analyzed statistically.

Conclusions made are based upon the probability of the existence or non-existence of differences as found by the application of Student's ('08) method of analyzing paired data. A difference between two averages was judged to be significant when the value of P was 0.03 or less.

RESULTS

Digestibility of the nitrogen and of the energy, and the metabolizable energy of diets containing different percentages of protein. Table 1 gives the average nitrogen and energy contents of the diets used. In table 2 are summarized the data obtained from the balance experiments. These data seem to justify the following conclusions: increasing the percentage of whole egg protein from 4 to 54, by direct substitution for starch in the diet: 1) increased the gross energy; 2) increased the percentages of gross energy and of nitrogen ingested that appeared in the urine; 3) increased the metabolizable energy but slowly; 4) had no effect upon the percentage of gross energy which was metabolizable until 30% or more protein was present, when a gradual decrease occurred; 5) caused a gradual decrease in the ratio of energy to nitrogen in the urine, and 6) had no consistent effect upon the digesti-

TABLE 1

Average composition of diets

Approximate protein content	*Total nitrogen*	*Crude protein (T.N. × 6.25)*	*Gross energy*
%	%	%	*Calories per gram*
4	0.668	4.17	4.641
8	1.315	8.22	4.722
12	1.908	11.93	4.752
16	2.553	15.96	4.846
18	2.851	17.82	4.800
20	3.259	20.37	4.848
22	3.549	22.18	4.886
30	4.688	29.30	5.083
42	6.647	41.54	5.238
54	8.684	54.28	5.480

TABLE 2

Energy and nitrogen utilized and wasted from the various diets

Approximate protein content, %	*4*	*8*	*12*	*16*	*20*	*30*	*42*	*54*
Energy								
Gross, Calories per gram of diet	4.641	4.722	4.752	4.846	4.848	5.083	5.238	5.480
Metabolizable,[1] Calories per gram of diet	4.412	4.382	4.429	4.606	4.624	4.702	4.754	4.842
S[2]	0.094	0.053	0.022	0.007	0.023	0.028	0.017	0.051
Metabolizable,[3] Calories per gram of diet	4.376	4.345	4.363	4.505	4.532	4.560	4.598	4.711
S	0.106	0.043	0.024	0.013	0.019	0.019	0.016	0.090
Metabolizable,[1] percentage of gross	95.2	94.4	93.6	94.4	93.4	91.6	90.5	87.9
S	0.50	0.40	0.16	0.21	0.49	0.55	0.30	0.80
Digestible, percentage of gross	96.4	95.8	95.3	96.3	96.0	95.3	95.1	94.3
S	0.4	0.4	0.2	0.1	0.2	0.2	0.2	0.4
Fecal, percentage of gross	3.62	4.16	4.67	3.70	3.97	4.66	4.99	5.66
S	0.44	0.37	0.18	0.17	0.23	0.17	0.16	0.41
Urinary, percentage of gross	1.14	1.43	1.68	1.93	2.62	3.71	4.50	6.41
S	0.27	0.37	0.15	0.06	0.21	0.51	0.25	0.77
Nitrogen								
Digested, apparent, %	75.9	81.4	80.0	90.5	88.5	90.9	92.5	88.1
S	3.9	1.7	2.8	0.5	2.2	0.6	0.2	4.0
Digested, true,[4] %	100[4]	93.5	88.7	96.9	93.7	94.5	94.9	90.0
Fecal, percentage of N intake	24.1	18.5	19.9	9.54	11.48	9.06	7.54	11.93
S	4.0	1.71	2.81	0.52	2.18	0.64	0.24	4.00
Urinary, percentage of N intake	57.3	46.1	35.0	38.4	50.8	68.4	61.4	67.9
S	13.5	13.1	2.80	4.38	8.3	3.5	3.0	7.1
Energy per gram N in urine, Calories	12.88	10.56	11.53	9.39	7.93	5.90	5.64	6.01
S	2.73	2.11	0.60	1.03	0.79	0.83	0.70	0.80
Number of determination	16	12	4	8	8	8	8	8

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[1] Uncorrected for nitrogen balance.

[2] $S = \sqrt{\frac{(A - X)^2}{n - 1}}$

[3] Corrected for nitrogen balance.

[4] Assuming the protein of the 4% diet to be completely digested. The average fecal N per gram of 4% diet was 1.92 mg. and this was assumed to be the metabolic N per gram on all other diets.

bility of either food energy or food nitrogen. It may be calculated that, increasing the protein in the diet from 4 to 54%, the gross energy increased 15.3% while the metabolizable energy increased but 8.8%. The per cent of the gross energy which was metabolizable decreased less than 8%, the greatest decrease occurring between the 30 and 54% protein diets.

The effect of the percentage of protein in the diet upon its growth-promoting value. The data relative to the growth-promoting value of the various diets are summarized in table 3. Using nitrogen retention as a measure of the growth-promoting abilities of the diets, the data show that, as the percentage of protein increases from 4 to some percentage between 12 and 16, the growth-promoting values of the diets also increase. Increasing the percentage of protein in the diet from 16 to 54 caused no further increase in protein retention by rats increasing in weight from 50 to about 200 gm.

The final body lengths of paired rats, gaining in weight at the same rate, increased with the percentage of protein in the diet until 12% was present, remained equal for diets containing between 12 and 42% protein and then decreased when 54% protein was present.

Using the amounts of metabolizable energy required to produce equal rates of gain in paired rats as a measure, the growth-promoting abilities of the diets increased with the percentage of protein from 4 to between 12 and 16, remained constant between 16 and 22 and then decreased when 42% or more protein was present. Using the ratio of total metabolizable energy intake of the low-protein-fed rats divided by that of the high-protein-fed rats making the same daily gains, the efficiencies of the various diets may be expressed as follows:

4 versus 8	1.220 : 1	16 versus 20	1.003 : 1
8 versus 12	1.155 : 1	18 versus 22	1.006 : 1
8 versus 18	1.207 : 1	22 versus 42	0.946 : 1
12 versus 16	1.029 : 1	20 versus 54	0.916 : 1

The minimum percentage of whole egg protein, capable of producing maximum growth, seems to lie between 12 and 16.

TABLE 3

The growth-promoting ability of diets containing different percentages of protein (averages for at least eight pairs of rats)

	4 versus 8[1]		*8 versus 12*		*8 versus 18*		*12 versus 16*		*16 versus 20*		*18 versus 22*		*22 versus 42*		*20 versus 54*	
Total food consumed—																
Gm.	511	417	886	776	598	480	641	607	475	469	421	413	466	469	411	415
P[2]	<0.0001		<0.0001		<0.0001		0.0001		0.19		0.10		0.32		0.23	
Total gains—																
Gm.	42.5	43.5	188.0	189.9	154.4	152.4	180.0	180.8	163.3	161.6	154.0	153.6	153.0	152.0	140.8	136.1
Final body length—																
mm.	162.9	165.5	215.8	217.6	208.8	209.1	216.0	217.3	209.6	212.0	208.0	208.5	209.1	210.9	208.4	205.9
P	0.0002		0.056		0.037		0.19		0.073		0.24		0.085		0.030	
Total metabolizable energy intake[3]—																
Cals.	2225	1824	3931	3402	2675	2217	2820	2740	2148	2142	1909	1898	2133	2249	1920	2095
P	<0.0001		<0.0001		<0.0001		0.0088		0.42		0.37		0.0041		0.0092	
Total nitrogen gained—																
Gm.	1.44	1.70	5.76	6.19	4.56	4.92	5.34	5.51	5.12	5.17	4.76	4.69	4.73	4.62	4.86	4.72
P	<0.0001		0.0004		0.0013		0.038		0.33		0.30		0.16		0.14	
Energy gained per gram N gained—																
Cals.	87.2	62.2	101.0	88.4	117.1	97.8	110.5	102.0	104.0	98.7	99.1	100.5	109.4	105.1	66.6	61.6
P	<0.0001		0.0012		0.0046		0.0048		0.18		0.4		0.29		0.0036	

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[1] Approximate percentage of protein in the diets compared.

[2] Probability that the difference between the averages was due to chance, according to 'Student,' ('08). A P-value of 0.03 or less is considered highly significant.

[3] Corrected to exactly equal gains.

While the data for the entire feeding period of the 16 versus 20% protein diets indicated that the 20% diet was no better than the 16, the daily food records indicated very strongly that, until the rats weighed about 125 gm., the 20% protein diet had a higher growth-promoting value than the 16. For very young rapidly growing rats, the minimum percentage of a high quality protein capable of supporting a maximum growth rate is probably near 20%. As to the maximum percentage of egg protein capable of producing maximum growth, 54% seems to be excessive. Although in the 22 versus 42% protein comparison pair-mates made the same gains in total weight, body length, nitrogen and energy on the same amounts of food, more metabolizable energy was required by the 42% protein-fed rats than was required by their 22% protein-fed pair mates. Also, as will be shown in the following paper, the heat increment of the 42% protein diet is greater than that of a 20% protein diet, while the heat increment of a 30% protein diet was no greater than that of a 20% protein diet. It seems, therefore, that the upper limit of whole egg protein capable of supporting a maximum rate of growth in rats is between 30 and 42%.

In general, the effects of the percentage of protein in the diet upon its growth-promoting ability, as reported here, are in agreement with the findings of other workers. It should be emphasized that the range of protein percentages over which a diet will be balanced will depend upon the nature of the protein and the requirements of the animal.

The effect of the percentage of protein in the diet upon the composition of the gains. In table 4 are summarized the data relative to the effects of the percentage of protein in the diets upon the composition of gains in rats. The compositions of the gains were calculated from the chemical analyses of the rats at the end of the feeding tests and from the composition of control rats slaughtered at the time the feeding test was started.

Data were obtained on eleven series of rats. In each series, eight or more pairs of rats were used. In three series, the

TABLE 4

The effect of the percentage of protein in the diet upon the composition of gains in rats. (Figures represent data from at least eight pairs of rats)

Diets compared	*Total gains*	*Total nitrogen gained*				*Total energy gained*				*Energy gained per gram T.N. gained*			
	gm.	gm.	M	s	P	Calories	M	s	P	Calories	M	s	P
4 (= food)	33.1	0.850				140.9				165.7	86.5	30.6	0.0001
8	67.0	2.314	1.464	0.413	<0.0001	183.3	42.4	36.3	0.0088	79.2			
8 (= food)	67.2	2.577				192.8				74.8	10.6	2.54	<0.0001
12	79.3	3.154	0.577	0.312	0.0009	202.6	9.8	17.4	0.092	64.2			
12 (= food)	85.3	2.991				288.7				96.5	4.0	11.2	0.19
16	88.4	3.244	0.253	0.122	0.0005	300.2	11.5	30.6	0.184	92.5			
4 (= gain)	42.5	1.443				125.8	20.1	18.7	0.013	87.2	25.0	6.57	<0.0001
8	43.5	1.699	0.256	0.069	<0.0001	105.7				62.2			
8 (= gain)	188.0	5.757				581.4	34.3	43.8	0.039	101.0	12.6	7.17	0.0012
12	189.9	6.187	0.430	0.202	0.0004	547.0				88.4			
8 (= gain)	154.4	4.564				543.2	52.6	47.9	0.011	117.1	19.3	14.3	0.0046
18	152.4	4.922	0.358	0.207	0.0013	481.6				97.8			
12 (= gain)	180.0	5.345				590.4	28.2	27.1	0.014	110.5	8.5	6.33	0.0048
16	180.8	5.511	0.166	0.211	0.038	562.2				102.0			
16 (= gain)	163.3	5.120				532.6	27.8	44.1	0.071	104.0	5.3	13.2	0.18
20	161.6	5.166	0.046	0.271	0.33	504.8				98.7			
18 (= gain)	154.0	4.756	0.062	0.297	0.30	471.2				99.1			
22	153.6	4.694				471.7	0.5	39.0	0.42	100.5	1.4	13.4	0.40
22 (= gain)	153.0	4.734	0.114	0.254	0.16	517.7	32.3	50.2	0.084	109.4	4.3	16.4	0.29
42	152.0	4.620				485.4				105.1			
20 (= gain)	140.8	4.856	0.135	0.246	0.14	277.7	26.7	21.8	0.0025	66.6	5.0	4.29	0.0036
54	136.1	4.721				251.0				61.6			

diets used varied in their percentage of protein from 4 to 16, and pair-mates were fed equal amounts of feed daily. In eight series, in which the protein contents of the diets varied from 4 to 54%, the pair-mates were fed such amounts of feed that equal daily gains were obtained. Data of similar significance were obtained from both types of feeding, although it seems reasonable to suppose that the data obtained from the equal gain procedure have the more exact significance in this connection because the pair-mates at all times throughout the test were of the same weight as well as of the same sex, age and parenthood.

From the data presented in table 4, the following conclusions seem justified: from 4 to 16% dietary protein the gains of rats on the higher plane of protein intake contained more nitrogen, while the gains of rats, increasing in weight at the same rate on a lower plane of protein intake, contained more fat. Diets containing from 16 to 42% protein produced gains of similar composition. In the 20 versus 54% protein comparison, while there were no significant differences in the amounts of nitrogen gained by pair-mates, the 20% protein-fed rats put on gains which were fatter.

The effect of the percentage of protein in the diet upon the appetite and the activity of the animal. In all paired-feeding tests records of daily food refusals were kept and in this manner evidence as to the relative effects of the diets upon the appetites of the rats was obtained. In three comparisons by the equal-daily-food technic and in four comparisons by the equal-daily-gain procedure, the amounts of food eaten by pair-mates daily were either exactly equal or approximately equal. The data in table 5 indicate a direct correlation between the growth-promoting values of the diets and their appetite stimulating effects. In all comparisons with diets containing less than 16% protein, the diet having the higher protein content also had the greater appetite stimulating effect. Diets containing between 16 and 22% protein were not only of equal growth-promoting values, but were also equal with respect to their effects upon the appetites of the rats.

TABLE 5

Effect of the percentage of protein in the diet upon the appetite of the animal

	4 versus 8[1]		*8 versus 12*[1]		*12 versus 16*[1]		*16 versus 20*[2]		*18 versus 22*[2]		*22 versus 42*[2]		*20 versus 54*[2]	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Total food consumed														
Gm.	731	731	335	335	273	274	475	469	421	413	466	469	411	415
P[3]	<0.0001		<0.0001		<0.0001		0.19		0.10		0.32		0.23	
Diet having the higher growth-promoting value	8		12		16		equal		equal		22		20	
Average number of food refusals	81.1	4.0	22.8	7.8	12.1	4.3	2.4	5.4	10.5	14.2	6.9	28.9	1.5	33.6
P[3]	<0.0001		0.029		0.024		0.16		0.16		0.0038		0.0002	

NOTE: The average figures in this table represent eight animals.

[1] Equal-daily-food procedure.

[2] Equal-daily-gain procedure.

[3] Probability that the difference was significant, according to 'Student' ('08).

In the 22 versus 42 and the 20 versus 54% protein comparisons, the higher protein diet in each case markedly depressed the appetites of the rats, and also in each case the higher protein diet was of less value for growth purposes. While these results are in agreement with the frequent assumption that the appetite of an experimental animal is an infallible index to the nutritive completeness of a diet, it should be pointed out that this is not always the case.

TABLE 6

The effect of the percentage of protein in the diet upon the activity of the animals consuming it. (Figures represent thousands of revolutions)

Pair Number	*4 versus 8*[1]		*8 versus 12*[1]		*20 versus 54*[2]	
	Low	High	Low	High	Low	High
1	230.0	413.4	431.1	371.7	234.5	105.0
2	447.6	487.8	347.3	379.7	131.0	105.5
3	504.2	455.6	235.1	353.3	208.9	251.4
4	429.4	198.2	391.9	137.6	268.4	83.6
5	319.4	262.6	45.8	418.0	239.4	166.5
6	267.4	378.5	272.0	232.0	266.7	117.5
7	323.2	437.3	226.8	459.7	170.2	173.2
8	455.5	433.8	389.6	327.9	272.6	247.6
Average	372.1	382.0	292.4	335.0	224.0	156.2
M		9.9		42.6	67.8	
S		122		183	74.9	
P		0.4		0.282	0.023	

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[1] Equal food daily.

[2] Equal gain daily (also approximately equal food).

In order to determine whether or not the percentage of protein in the diet affected the voluntary activity of the rats, three series of paired rats were kept for several weeks in a battery of sixteen identical revolving cages, each cage being equipped with a revolution counter. The three series in which the voluntary or running activity was measured were the 4 versus 8 (= food), 8 versus 12 (= food) and the 20 versus 54 (= gain) % protein diets. The data are given in table 6. According to these data, percentages of protein varying from 4 to 12 had no differential effect upon the running activity of the rats. It therefore seems unlikely that diets better balanced with respect to protein would do so. Rats receiving a 54% protein diet, however, were less active than rats growing at the same rates on approximately the same amounts of a 20% protein diet.

Forbes, Swift, Black and Kahlenberg ('35 b), working also on the problem of food energy utilization as affected by the percentage of protein, attempted to explain their results on the assumption of a decreased voluntary activity of the rats as the diets became better balanced with respect to protein. The data reported here do not support this assumption.

TABLE 7

The effect of the percentage of protein in the diet upon the weights of the livers and kidneys of the rats consuming it

	LIVER WEIGHTS		KIDNEY WEIGHTS					
	20 versus 54 (= gain)		8 versus 18 (= gain)		22 versus 42 (= gain)		20 versus 54 (= gain)	
	Low	High	Low	High	Low	High	Low	High
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Average	7.25	8.04	1.47	1.63	1.65	1.87	1.59	1.83
M		0.79		0.16		0.22		0.24
S		0.60		0.128		0.12		0.168
n		11		8		8		11
P		0.0006		0.0066		0.0009		0.0005

The effect of the percentage of protein in the diet upon the liver and kidney weights of animals consuming it. That high protein diets produce hypertrophy of the kidneys of the animals consuming them is well established, although the effects upon the liver are less certain. In most previous work of this kind, comparisons were made with so-called normals. In this study, having at hand a number of rats of the same age, sex, size, parenthood and having grown at the same rate on diets which differed only in their contents of protein, it seemed that conditions were ideally controlled to study the effects of the percentage of protein in the diet upon liver and kidney weights. The data obtained upon this point are summarized in table 7, and from these data it is concluded that there is a direct relationship between the percentage of protein in the diet of growing rats and the weights of their livers and kidneys.

CONCLUSIONS

1. Increasing the percentage of whole egg protein from 4 to 54 by the direct substitution of starch in the diet of rats 1) increased the metabolizable energy but slowly; 2) had no significant effect upon the percentage of gross energy which was metabolizable until 30% or more protein was present when a gradual decrease occurred; 3) caused a gradual decrease in the ratio of energy to nitrogen in the urine, and 4) had no consistent effect upon the digestibility of either food energy or food nitrogen.

2. Increasing the percentage of protein from 4 to 16% in the diet of growing animals, increased the growth-promoting value of the diet. Diets containing between 16 and 30% of a good quality protein were of equal growth-promoting value, and as the protein increased above 30%, the growth-promoting value decreased. For male rats weighing less than 125 gm., the minimum percentage of protein in a well-balanced diet may be as high as 20.

3. Total gains in weight are not as good a criterion of growth as is gain in protein and gain in body length.

4. The appetites of rats consuming diets containing less than 16 or more than 22% protein were adversely affected.

5. The voluntary activity of rats was unaffected by the protein content of the diet until an excess was present when the activity was decreased.

6. The composition of the gains of growing animals was readily affected by the percentage of protein in the diet. Diets containing less than 16% protein produced gains having more fat but less protein than did well-balanced diets having a higher percentage of protein. Diets containing between 16 and 42% protein produced gains of approximately the same composition. A high protein (54% whole egg protein) diet produced gains with less fat than did a 20% protein diet.

7. The weights of the kidneys and the livers of growing rats are influenced by the amounts of protein consumed, the relationship being a direct one.

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LITERATURE CITED

BEADLES, JESSIE R., W. W. BRAMAN AND H. H. MITCHELL 1930 The cystine deficiency of the proteins of garden peas and of potatoes. J. Biol. Chem., vol. 88, pp. 615–622.

FORBES, E. B., R. W. SWIFT, A. BLACK AND O. J. KAHLENBERG 1935 a The plane of protein intake as affecting food utilization. Proc. Am. Soc. Animal Prod., 1934, pp. 181–182.

——— 1935 b The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. III. The effects of the plane of protein intake. J. Nutrition, vol. 10, pp. 461–479.

FORBES, E. B., LEROY VORIS, J. W. BRATZLER AND WALTER WAINIO 1938 The utilization of energy producing nutriment and protein as affected by the plane of protein intake. J. Nutrition, vol. 15, pp. 285–307.

HAMILTON, T. S. 1935 Facors affecting the thermogenic effect of food. Rpt. of Conference on Energy Metabolism held at State College, Pa., June 14–15, 1935. Nat'l Res. Council Mimeograph Rep.

——— 1937 The influence of the percentage of protein upon the thermogenic (specific dynamic) effect and the net energy of the diet. (Abstract.) Proc. Am. Inst. Nutrition; J. Nutrition, vol. 13, p. 16. (Presented at Memphis, Tennessee, April 21, 1937.)

HOGAN, A. G., S. R. JOHNSON AND U. S. ASHWORTH 1935 Rate of growth in relation to the plane of protein intake. Proc. Am. Soc. Animal Prod., 1934, pp. 179–181.

HOGAN, A. G., AND R. W. PILCHER 1933 Effects of variations in the amounts of vitamin B and protein in the ration. Missouri Agr. Exp. Sta. Res. Bull. 195.

JOHNSON, S. R., A. G. HOGAN AND U. S. ASHWORTH 1936 The utilization of energy at different levels of protein intake. Missouri Agr. Exp. Sta. Res. Bull. 246, 62 pp.

MITCHELL, H. H. 1932–1933 Ill. Agr. Exp. Sta. Ann. Rep., p. 75.

MITCHELL, H. H., AND JESSIE R. BEADLES 1930 The paired-feeding method in nutrition experiments and its application to the problem of cystine deficiencies in food proteins. J. Nutrition, vol. 2, pp. 225–243.

MITCHELL, H. H., AND T. S. HAMILTON 1936 The balancing of rations with respect to protein. Proc. Am. Soc. Animal Prod., 1935, pp. 241–252.

MITCHELL, H. H., AND D. B. SMUTS 1932 Amino acid deficiencies of beef, wheat, corn, oats and soybeans for growth in the white rat. J. Biol. Chem., vol. 95, pp. 263–281.

SLONAKER, J. R. 1931 Effect of different per cents of protein in the diet. II. Spontaneous activity. Am. J. Physiol., vol. 96, pp. 557–561.

'STUDENT' 1908 The probable error of a mean. Biometrika, vol. 6, pp. 1–25.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, vol. 75, pp. 339–340.

THE HEAT INCREMENTS OF DIETS BALANCED AND UNBALANCED WITH RESPECT TO PROTEIN [1]

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INTRODUCTION

That the nutritive balance of a diet was a determining factor in the efficiency with which an animal utilizes the dietary energy, was first recognized, so far as the writer is aware, by Møllgaard ('23). He stated, "The quotient of production (the proportion of the net energy of the ration contained in the protein component) of any feeding-stuff is substantially determining the extent to which the net energy of the feeding-stuff may be utilized in production of milk."

A clear and definite statement of this relationship was made by Mitchell ('24) who, in commenting upon some experiments of Osborne and Mendel, stated (loc.cit., footnote, p. 447) "These experiments suggest an interesting relation, hitherto uninvestigated as far as the author is aware, between the balance of nutrients in a ration and the utilization of its energy." The same idea was again expressed by Mitchell and Carman ('26) who obtained as much as 40 to 50% greater gains on the same amount of food in both chicks and rats by substituting 1% sodium chloride for 1% corn in a salt-deficient ration. They stated (loc.cit., p. 177): "The growth data of this experiment afford a striking demonstration of the fact that the utilization of food energy by growing animals may be greatly impaired by an improper balance among indispensable dietary factors. The addition of 1% NaCl to ration A has evidently greatly improved the utilization of its available energy, probably by decreasing its stimulating effect on heat production, i.e., its specific dynamic effect, and possibly to some extent by decreasing the muscular activity or restlessness of the rat itself."

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[1] The experimental data reported here have been taken from a thesis submitted in 1937 to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy in animal husbandry. Preliminary reports of this investigation have appeared previously (Hamilton, '35, '37).

In 1928 appeared the first of a series of important papers dealing with and apparently developing independently a similar conception by Forbes and associates. The authors of this first paper were Forbes, Braman and Kriss ('28).

In the second paper of this series, Forbes, Braman and Kriss ('30) made the following statement (loc.cit., p. 65): "In the light of these facts it may be there are, in reality, different net-energy values of feedingstuffs—for growing animals at least—in accord with different nutritive ratios in which the feedingstuffs are combined." And confirmation of this idea was obtained by Forbes, Braman, Kriss and Swift ('31) when they found that the heat increment of corn fed alone at a maintenance level was much greater than that indirectly computed for corn fed at the same level in combination with alfalfa hay. Similar evidence was presented by Forbes, Braman, Kriss and Swift ('33). Other papers concerned with the development of this idea by the Pennsylvania workers have appeared under the authorship of Forbes ('29, '33 a, '33 b); Forbes and Kriss ('32 a, '32 b, '32 c); and by Forbes, Kriss and Miller ('34).

Other workers also have apparently and independently arrived at the conclusion that the utilization of dietary energy is increased by improving the balance of the nutrients in the diet. Thus Fraps ('31, p. 26) states: "The addition of a proteid feed to an unbalanced ration increases the utilization of the energy of the entire ration. The productive energy of a feed in an unbalanced ration is lower than it is in a balanced ration," and "A proteid feed added to an unbalanced ration has an effect greater than its own productive energy, since it increases the utilization of the other feeds to which it is added. In 1929, Møllgaard ('29, p. 165) presented evidence in confirmation of his 1923 conception when he found that the utilization of dietary energy for milk production was greatest when the ratio of protein energy to carbohydrate and fat energy in the ration approximated the corresponding ratio in milk, i.e., between 0.14 and 0.23. Hansson ('29, p. 53) calculated the relative nutritive values of protein for fattening and for milk production and found that 1 kg. protein for fattening had a starch value of 0.94, while for milk production 1 kg. protein had a starch value of 1.43 kg.

Further evidence is afforded by numerous controlled-feeding experiments in which greater utilization of the energy of diets better balanced with respect to salt, phosphorus, vitamin B, vitamin A, vitamin D, vitamin G, cystine, lysine and protein has been observed. (References to these investigations may be found in a paper by Hamilton, '35.)

Observations of uncommonly low or decreased 'specific dynamic effect' of nutrients or diets, when ingested by animals whose requirements for these nutrients or diets are intensified, are of similar significance. Numerous such observations may be found in the literature (compare Hamilton, '35) and while many lacked proper control, the mass of such observations constitutes strong evidence that the specific dynamic effects of separately ingested nutrients or the heat increments of diets are not constant characteristic values but are variable —decreasing as the metabolic mixture more nearly satisfies the animal's requirements.

Richardson and Mason ('23) obtained some interesting results in this connection. Working with diabetics, they found that a mixed diet, containing the same quantities of protein, fat and carbohydrates calculated to have been oxidized under basal conditions, if divided into portions and administered every 2 hours, produced no increase in the heat production over the basal level. Concerning the significance of these results, Lusk ('31) in one of his last papers said, "We may conclude from this evidence that, if a mixed diet be so constituted as to conform to the exact needs of the tissues and be slowly introduced by absorption from the intestines, its specific dynamic action is virtually negligible . . . .''

Recent studies concerned with the effect of the amount of food consumed upon the utilization of its energy have demonstrated that the heat increment per unit of food is not constant but dependent, at least over certain ranges, upon the amount of food consumed. Experiments of this nature have been reported by Gigon ('11) in a human subject, by Forbes, Braman and Kriss ('28, '30) and by Mitchell and Hamilton ('32) in steers, by Wiegner and Ghoneim ('30) in a rabbit and by Forbes, Kriss and Miller ('34) in rats.

The effect of the percentage of protein in the diet upon the utilization of its dietary energy, has been reported by Johnson, Hogan and Ashworth ('36), Forbes, Swift, Black and Kahlenberg ('35) and Forbes, Voris, Bratzler and Wainio ('38) in rats; by Mitchell and Hamilton ('36) in pigs, and by Hammond, Hendricks and Titus ('38) in chickens.

The purpose of the experiments to be reported here was to test the validity of a conception, definitely stated by Mitchell ('34), that the better balanced a ration is in satisfying an animal's requirements, the smaller will be its heat increment and the greater will be its net energy value. Specifically, this series of experiments was concerned with the heat increments and the net energy contents of diets containing varying percentages of protein when fed to satisfy the requirements of growth in young albino male rats.

PLAN OF INVESTIGATION

The general plan of this investigation, which was started in July, 1934, consisted of a series of experiments on white rats, involving paired-feeding, carcass analyses, metabolism tests and determinations of the respiratory exchange during both the absorptive and post-absorptive states. The experimental diets were complete and alike in all respects, except for the percentage of protein which was varied from 4 to 54.

The protein was supplied in the form of dried, extracted whole egg and increases in the percentages of protein were accomplished at the expense of cornstarch. While this procedure had the effect of gradually increasing the gross energy of the diets as the percentage of protein was increased, the metabolizable energy increased but slowly (compare preceding paper).

All tests were of a comparative nature, and the various diets were compared in a number of respects. In the preceding paper, the effects of the percentage of protein in the diet upon the growth-promoting value, the digestibility of the nitrogen and energy and the metabolizable energy contents of the diets, the appetites, activity and composition of the gains in rats were reported. The data to be reported here are concerned with the effects of the percentage of protein in the diet upon its heat increment and net energy value when fed to growing rats. The diets studied contained the following approximate percentages of protein (T.N. × 6.25): 4, 8, 12, 18, 20, 30, 42 and 54. Respiratory exchange of rats during both the absorptive and post-absorptive states was made on all rats in the following comparisons: 4 versus 8, 4 versus 12, 8 versus 18, 20 versus 30, 20 versus 42 and 20 versus 54% protein diets. Eight pairs of rats of the same age, sex and parenthood were used in most comparisons. Details of diets, methods of feeding, housing, etc. are described in the preceding paper.

The rats were housed in small four-compartment wire cages in order to restrict their activity, and it was hoped also that the activity of pair-mates would be more nearly equal. All rats were kept in an air-conditioned room, the temperature of which was maintained at 28 ± 1°C. and the humidity at 43 ± 5%. All respiration studies were also carried out in this same room. The method of feeding, which was adopted in all series reported here, was a modified paired-feeding technic in which pair-mates were fed, not necessarily the same amounts of food daily, but such amounts that equal daily gains resulted. This procedure has the advantage that since pair-mates were always of the same weight, their maintenance requirements would be approximately the same, and, in addition, heat productions could be compared directly; or if comparisons were made on the basis of body surface, the uncertainties of any surface area formula would be negligible.

The heat increment, as used in this study, was the heat production above the basal for 17 to 19 hours immediately following the ingestion of 5 gm. (4 in a very few cases) of diet. The basal heat production of each rat was determined in a 3- to 5-hour test which followed the heat increment test. The basal tests were therefore begun after the rat had fasted at least 19 hours. All basal tests were run between the hours of 11 A.M. and 5 P.M. All rats were fasted 24 hours previous to the test meal offered in the heat increment test, and the rat was allowed a maximum of 15 minutes in which to consume entirely the test meal.

The Haldane (1892) gravimetric procedure was used for the heat increment tests and the Benedict ('30) multiple-unit respiration apparatus, modified so as to measure the CO_2 production gravimetrically, was employed in the basal tests. In both types of apparatus, a kymographic record of activity was obtained, and all experiments in which evidence of more than minor movements of the rat were obtained were repeated.

The heat increments of diets containing 4, 8, 18, 20 and 54% protein were determined, using rats that had been on a paired-feeding test for at least 3 weeks. Only three pairs of rats in the 4 versus 8% protein series would consume sufficient food in the allotted 15 minutes to make a satisfactory test, although eight pairs were used in all other tests. The heat increments of diets containing 4, 12, 20, 30 and 42% protein were also determined using male rats weighing between 125 and 150 gm. and maintained on a good stock diet for 7 to 14 days. The comparisons made in this latter series were the 4 versus 12, 20 versus 30 and 20 versus 42% protein diets. In making these comparisons, the heat increments of both diets being compared were made on each of eight rats, and on alternate rats the order in which the diets were tested was reversed.

RESULTS

The effect of the percentage of protein in the diet upon its heat increment. In table 1 are summarized the data concerned with the influence of the percentage of protein in the diet upon its heat increment. In all except two cases the data represent averages from eight pairs of rats. Average differences and probabilities of their significance, according to 'Student's' ('08) method, are also given.

TABLE 1

The effect of the percentage of protein in the diet upon the heat increment

Protein in diets compared %	*Average heat increment Small calories per gram*	*M*	*S*	*N*	*P*
4	415	111	33	3	0.021
8	304				
4	330	95	95	8	0.017
12	235				
8	500	365	205	8	0.0010
18	135				
20	94				
30	158	64	116	8	0.12
20	232				
42	492	260	191	8	0.0044
20	152				
54	625	473	361	8	0.0047
54 (Cooked)	784			4	

Note: The heat increment is the total increase, above the basal, in the heat production of rats for a 17- to 19-hour period immediately following the consumption of 5 gm. of the experimental diet. (To a few pair-mates 4 gm. of diet were offered.)

Reference to the table will show that, in all three comparisons made within the range of 4 to 18% protein, the diet containing the lower percentage of protein had the higher heat increment. Diets containing 20 and diets containing 30% protein produced about the same heat increment, and the values for these two diets were very low and of about the same magnitude as the 18% protein diet. Diets containing 42% or more protein caused increasingly greater heat increments when fed to growing rats.

As the standard deviations indicate, rather wide differences in the heat increments between pair-mates in the same series were sometimes obtained. This is to be expected since very small differences in total heat production were being measured, over 90% of the total heat produced in a test being accounted for by the basal metabolism. However, it was extremely gratifying to obtain differences between pair-mates in a series almost always in the same direction. The individual data for each animal in two different series of tests presented in table 2 will illustrate this point.

TABLE 2

Heat increments of the 4 and 12% protein diets, each fed to the same rat, and the heat increments of the 20 and 42% protein diets fed to paired rats (results expressed in small calories per gram of diet)

Identical rat series[1]			*Paired-rat series*		
Rat number	4% protein	12% protein	Pair number	20% protein	42% protein
469	579	269	1	389	720
470	357	246	2	239	439
471	251	99	3	0	632
472	479	464	4	64	490
473	289	261	5	238	522
474	262	251	6	261	317
475	268	158	7	415	436
476	158	133	8	246	378
Average	330	235		232	492
M	95				260
S	95				191
P	0.017				0.0044

[1] The heat increment of the diets used in this test were made on successive days on the same rat, and the order in which the diets were fed was reversed with alternate rats.

Note: Because of lack of space, these are the only individual data presented. The writer will loan copies of the individual data from which the summary tables presented in this publication are based to any one requesting them.

Because of a number of possible factors (feed level, previous feed level, composition of the animal, B.M.R., etc.), the most accurate and significant comparisons are those in a single series where controls were most exact and comparable. It is interesting, nevertheless, to calculate the average heat increments of the diets used. This has been done by simply averaging all heat increments obtained on each diet regardless of whether or not they were obtained in different series. These averages, together with the average gross, metabolizable and net energy contents of the various diets, are given in table 3.

TABLE 3

Average heat increments and net energy values of diets containing different percentages of protein

Per cent protein in diet	*Gross energy* [1] Cal.	*Metabolizable energy* [1] Cal.	*Net energy* [1, 3] Cal.	*Heat increment* [1] Cal.	*Heat increment* [2] %	*Net energy* [2] %
4	4641	4412	4040	372	8.43	91.57
8	4722	4382	3980	402	9.17	90.83
12	4752	4429	4194	235	5.30	94.69
18	4800	4507	4372	135	3.00	97.00
20	4848	4624	4465	159	3.44	96.56
30	5083	4702	4544	158	3.36	96.64
42	5238	4754	4264	492	10.35	89.69
54	5480	4842	4217	625	12.90	87.09

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[1] Small calories per gram.

[2] Expressed as a per cent of the metabolizable energy.

[3] Calculated by subtracting the average heat increment from the metabolizable energy.

The absolute values given in table 3 are of no great significance, except as they apply to the particular kind of protein used, the amounts of diet consumed and the particular requirements of the animals consuming the diet. The trends, however, are highly significant. For example, the average heat increment of the 8% protein diet was 402 calories per gram, a figure slightly higher than the average of 372 for the 4% diet; however, in the series in which the 4 and 8% protein diets were compared in comparable rats, the heat increments were 415 for the 4 and 304 for the 8% diet. Forbes, Voris, Bratzler and Wainio ('38) have recently criticized a previously published conclusion from this study (Hamilton, '35, '37) because the average heat increment of the 4% diet was slightly higher than that of the 8% protein diet. The writer believes that the above data show the criticism to be unjust.

As may be seen from the data in tables 1, 2 and 3, the heat increments of the diets fed to growing rats decreased as the percentage of protein increased up to about 18, remained practically constant for diets containing between 18 and 30% protein, then rapidly increased as the percentage of protein increased above 42. The average heat increment, expressed as a per cent of the metabolizable energy, was fairly high, about 9%, for diets containing between 4 and 8% protein; was very low, not much more than 3%, for diets containing between 18 and 30% protein, and was high again, nearly 13%, for the 54% protein diet. A similar trend in the percentage figures is obtained if the gross energy of the diets is used, but unquestionably the loss as heat increment related to the metabolizable energy content is more significant.

Because of some evidence of 'egg white' toxicity, particularly in the rats receiving the higher percentages of the commercial egg protein, the heat increment of a 54% cooked egg protein diet was determined, using four stock rats similar in age, sex and size to those used in previous tests. The heat increment of this cooked protein diet averaged 784 ± 133 calories per gram of diet—a value somewhat higher than the average found for the commercial egg protein diet. It may be assumed, therefore, that the high heat increment observed with the high protein diets was due to the disproportionate amount of protein to other nutrients in the diet, and not appreciably to egg white toxicity.

Comparing the growth-promoting properties of diets containing different percentages of protein with their heat increments, a remarkable inverse relationship is evident. Just as the growth-promoting values of these diets increased as the percentage of protein increased from 4 to at least 16, and then decreased when the diets contained 42% or more protein (compare preceding paper) the wastages of food energy as heat increment decreased as the percentage of protein increased from 4 to approximately 18 and then rapidly increased in diets with 42% or more protein.

In diets containing extracted dried whole egg as the source of protein and otherwise complete and adequate, the proper balance for the growing rat seems to be obtained when between 16 (probably 20 for young rats) and at least 30 (seemingly between 30 and 42) % protein is present. It is between these two levels that the diets showed the highest growth-promoting values and, at the same time, the smallest waste of energy as heat (heat increment).

The explanation of the effects of the percentage of protein in the diets used in this study upon its growth-promoting value now seems clear. When two diets, one less well balanced with respect to protein than the other, are fed to paired rats in equicaloric amounts, the rat receiving the better balanced diet grew faster and deposited more energy in body gains than did the rat on the less well balanced diet. The reason why the rat receiving the better balanced diet (the one which satisfied more completely the animal's requirements) deposited more energy in his gains than did the animal on an equicaloric amount of the less balanced diet was not because the animal was less active, not because the diet was digested more completely nor because the rat's basal metabolism had been lowered,[2] but primarily, and perhaps solely, because there was less wastage of absorbed energy as heat (heat increment).

The explanation as to why the heat increments of diets decrease instead of increase, as the percentage of protein in the diet increases from 4 or 8 to at least 16, cannot be found in any of the generally accepted theories of specific dynamic effect. Contrary to the present findings, all of the generally accepted theories of specific dynamic effect would predict an increase in the heat increment coincident with every increase in protein consumption, for regardless of several differences of opinion as to the physiological mechanism, protein has always been found to have had a much greater specific dynamic effect than either carbohydrates or fats.

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[2] The calculated energy used for basal metabolic purposes expressed as a per cent of the metabolizable energy intake was, for each series as follows: 4 versus 8 (= food), 68.7 and 78.4; 8 versus 12 (= food), 55.7 and 56.6; 12 versus 16 (= food), 50.3 and 50.7; 4 versus 8 (= gain), 68.4 and 76.8; 8 versus 12 (= gain), 64.8 and 71.8; 12 versus 16 (= gain), 59.6 and 58.6; 8 versus 18 (= gain), 55.1 and 64.3; 16 versus 20 (= gain), 60.5 and 59.6; 18 versus 22 (= gain), 54.9 and 55.2; 22 versus 42 (= gain), 54.2 and 52.0; and 20 versus 54 (= gain), 58.1 and 56.7.

Forbes, Swift, Black and Kahlenberg ('35) working also on the problem of food energy utilization as affected by the percentage of protein, found that the growth-promoting abilities of the diets increased as the percentage of protein increased from 10 to about 20 and they also calculated that, as the percentage of protein increased from 10 to 20, a decreasing amount of energy was either expended as energy of voluntary activity or lost as energy of heating effect (heat increment) (the plan of their experiment making it impossible to differentiate between the two). In an attempt to determine in which of these two ways the decrease was affected, these workers estimated the specific dynamic effects of their diets using previously determined specific dynamic effect factors for proteins, fats and carbohydrates when fed separately (Kriss, Forbes and Miller, '34). By this method they found, naturally, that as the percentage of protein in the diet increased, the estimated heating effect of the diets also increased. Since this was just the opposite of what would be necessary to explain their results, they assumed without measuring it that the activities of the rats must have been voluntarily suppressed sufficiently to account for the observed decrease in total heat production.

In the present study, both the voluntary activities and the heat increments of the diets were directly measured. It was found that as the per cent of protein in the diet increased from a low level to one adequate for rapid growth, the voluntary activity of rats does not decrease and, further, that the heat increments of the diets do decrease as the percentage of protein in the diets increases from a low level to levels adequate for rapid growth.

Concerning diets containing a sufficient excess of protein to lower their growth-promoting abilities, it was found that they had higher heat increments than did well-balanced diets. Also, the voluntary activities of rats receiving a 20% protein diet were greater, not smaller, than were those of rats receiving a 54% protein diet (compare preceding paper).

Forbes, Voris, Bratzler and Wainio ('38) in a recent contribution on this same problem found, in agreement with the present findings, a decrease in the growth-promoting values of diets containing an excess of protein (more than 25% according to Forbes and more than 30 according to the present findings), coincident with an increase in heat production. Again the Pennsylvania workers did not fractionate the energy contributing to the heat production but concluded that the decrease in the quantity of energy utilized for body gain, which is not a measure of total utilizable energy, was due to a more rapid decrease in metabolizable energy than in heat production.

From data presented in the preceding paper, it was concluded that, as the percentage of protein increases above 20, the percentage of the gross energy which was metabolizable, decreased. However, when the quantity of energy lost as heat (heat increment) was actually measured, it was found that the percentage of the metabolizable energy lost increased. For the 20% protein diet, it averaged 3.44; for the 42% protein diet, 10.35, and for the 54% protein diet, 12.90.

Johnson, Hogan and Ashworth ('36) have also studied the effects of different levels of protein intake upon the utilization of food energy. These workers found greater rates of gain by rats on diets containing 26% protein than on diets containing 10% protein. The plan of their experiments, however, enabled them to study the partition of the metabolizable energy only with respect to the energy stored in the gains. They report no difference in the total energy stored on the two diets, but, in agreement with others, that the rats on the low protein diet stored more fat and less protein than did the rats on the higher protein intake.

But the energy stored is only one way in which the metabolizable energy of a diet is utilized, and a measurement of the extent to which the metabolizable energy is retained in the body is not a complete measure of the extent to which the energy of a diet is utilized by the body.

The utilizable energy of a diet is that fraction of the metabolizable energy which can be used in supplying energy for 1) basal metabolism, 2) voluntary activity and 3) productive purposes. The summation of these fractions constitutes the net energy of the diet. An accurate comparison of the efficiency with which the energy of different diets is utilized cannot be made without including all three of these fractions. Since it is very difficult, if not impossible, to measure accurately the energy of voluntary activity, the most feasible method of estimating the net energy of a diet is by determining the heat increment and subtracting it from the metabolizable energy. This was the procedure followed in the investigation here reported.

The net energy, measured as the metabolizable energy minus the heat increment (equal to the total increase above the fasting level in the heat production incident to the consumption of the diet), was found to vary directly, and the heat increment inversely, with the growth-promoting values of the diets. These findings are in harmony with previous observations referred to above to the effect that the specific dynamic effects of the food nutrients were less when the requirements of the animals for the nutrients were greatest, and also that diets better balanced with respect to other essential nutrients will promote more rapid growth rates than will an equicaloric amount of less well-balanced diets.

The data support the conception that the better balanced a diet is in satisfying an animal's requirements, the smaller will be its heat increment and the greater will be its net energy value.

CONCLUSIONS

Increasing the percentage of protein from 4 to 54 by direct substitution of dried, extracted whole egg for starch, in the diet of growing male rats, had the following effects upon the utilization of the dietary energy:

1. The heat increment (specific dynamic effect) of the diet decreases coincident with every increase in the percentage of protein until about 18% is present; it remains constant in diets containing between 18 and at least 30% protein; and then increases again in diets containing 42% or more protein.
2. There is a remarkable inverse relationship between the heat increments and the growth-promoting abilities of the diets.
3. The net energy value of diets for growth plus maintenance increased as the percentage of protein in the diet increased up to about 16%, remained practically constant throughout the range of protein percentages over which the diets were equally well balanced (16 to 30%) and then decreased rapidly when an excess of protein was present (perhaps more than 30% whole egg protein).
4. The explanation of the higher net energy value of well-balanced diets, as compared with diets unbalanced either because of an insufficiency or because of an excess of protein, lies almost entirely in the smaller heat increment of the well-balanced diets.
5. The percentages of metabolizable energy used for basal expenditures or for total voluntary activity are unaffected by the percentage of protein in the diet, independent of the amount of food consumed.

The findings in this investigation support the general conception that the better balanced a diet is in satisfying an animals requirements, the smaller will be its heat increment and the greater will be its net energy value.

The writer is indebted to Prof. H. H. Mitchell for many valuable suggestions made throughout the progress of this investigation.

LITERATURE CITED

BENEDICT, F. G. 1930 A multiple-chamber respiration apparatus for rats and other small animals. J. Nutrition, vol. 3, pp. 161–176.

FORBES, E. B. 1929 The problem of determination of net-energy values of feedingstuffs. XIIIth International Physiological Congress. Abstracted in Am. J. Physiol., vol. 90, p. 348.

——— 1933 a Net energy of completely balanced nutriment as a measure of nutritive value of rations and of nutritive requirement of animals. Proc. Am. Soc. Animal Prod., 1932, pp. 32–40.

——— 1933 b The law of maximum normal nutritive value. Science, vol. 77, pp. 306–307.

FORBES, E. B., W. W. BRAMAN AND M. KRISS 1928 The energy metabolism of cattle in relation to the plane of nutrition. J. Agr. Res., vol. 37, pp. 253–300.

——— 1930 Further studies of the energy metabolism of cattle in relation to the plane of nutrition. J. Agr. Res., vol. 40, pp. 37–78.

FORBES, E. B., W. W. BRAMAN, M. KRISS AND R. W. SWIFT 1931 The metabolizable energy and net energy values of corn meal when fed exclusively and in combination with alfalfa hay. J. Agr. Res., vol. 43, pp. 1015–1026.

——— 1933 The associative effects of feeds in relation to the utilization of feed energy. J. Agr. Res., vol. 46, pp. 753–770.

FORBES, E. B., AND M. KRISS 1932 a The nutritive requirements of the dairy cow expressed in accord with a new method of application of the net-energy conception. Proc. Am. Soc. Animal Prod. 1931, pp. 113–120.

——— 1932 b The analysis of the curve of heat production in relation to the plane of nutrition. J. Nutrition. vol. 5, pp. 183–197.

——— 1932 c Der Energiewechsel des Rindes in Beziehung zur Grösse der Nahrungsaufnahme. Die Tierernährung, Bd. 4, S. 215–239.

FORBES, E. B., M. KRISS AND R. C. MILLER 1934 The energy metabolism of the albino rat in relation to the plane of nutrition. J. Nutrition, vol. 8, pp. 535–552.

FORBES, E. B., R. W. SWIFT, A. BLACK AND O. J. KAHLENBERG 1935 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. III. The effects of the plane of protein intake. J. Nutrition, vol. 10, pp. 461–479.

FORBES, E. B., LEROY VORIS, J. W. BRATZLER AND WALTER WAINIO 1938 The utilization of energy producing nutriment and protein as affected by the plane of protein intake. J. Nutrition, vol. 15, pp. 285–307.

FRAPS, G. S. 1931 Productive energy of feeds calculated from feeding experiments with sheep. Texas Agr. Exp. Sta. Bull. 436, 56 pp.

GIGON, A. 1911 Über den Einfluss der Nahrungsaufnahme auf den Gaswechsel und Energieumsatz. Arch. ges. Physiol., Bd. 140, S. 509–592.

HALDANE, J. 1892 A new form of apparatus for measuring the respiratory exchange of animals. J. Physiol., vol. 13, pp. 419–430.

HAMILTON, T. S. 1935 Factors affecting the thermogenic effect of food. Rep. of Conference on Energy Metabolism held at State College, Pa., June 14–15, 1935. Natl. Res. Council Mimeograph Rep.

HAMILTON, T. S. 1937 The influence of the percentage of protein upon the thermogenic (specific dynamic) effect and the net energy of the diet. (Abstract) (Proc. Am. Inst. Nutr.; J. Nutrition, vol. 13 (Suppl.), p. 16. (Presented at Memphis, Tenn., April 21, 1937.)

HAMMOND, J. C., W. A. HENDRICKS AND H. W. TITUS 1938 Effect of percentage of protein in the diet on growth and feed utilization of male chickens. J. Agr. Res., vol. 56, pp. 791–810.

HANSSON, N. 1929 Fütterung der Haustiere. Steinkopff, Dresden.

JOHNSON, S. R., A. G. HOGAN AND U. S. ASHWORTH 1936 The utilization of energy at different levels of protein intake. Mo. Agr. Exp. Sta. Res. Bull. 246, 62 pp.

KRISS, M., E. B. FORBES AND R. C. MILLER 1934 The specific dynamic effects of protein, fat and carbohydrate as determined with the albino rat at different planes of nutrition. J. Nutrition, vol. 8, pp. 509–534.

LUSK, GRAHAM 1931 The specific dynamic action. J. Nutrition, vol. 3, pp. 519–530.

MITCHELL, H. H. 1924 The nutritive value of proteins. Physiol. Revs., vol. 4, pp. 424–478.

———— 1934 Balanced diets, net energy values and specific dynamic effects. Science, vol. 80, pp. 558–561.

MITCHELL, H. H., AND G. G. CARMAN 1936 Does the addition of sodium chloride increase the value of a corn ration for growing animals? J. Biol. Chem., vol. 68, pp. 165–181.

MITCHELL, H. H., AND T. S. HAMILTON 1932 The effect of the amount of feed consumed by cattle on the utilization of its energy. J. Agr. Res., vol. 45, pp. 163–191.

———— 1936 The balancing of rations with respect to protein. Proc. Am. Soc. Animal Prod. 1935, pp. 241–252.

MØLLGAARD, H. 1923 New views regarding the scientific feeding of dairy cattle. Experiments in connection with the problem of the requirements of energy and matter for milk production, and investigations into the question of the possibility of a general unit for measuring the productive values of feedingstuffs. Compt. rend. Congress Internat. l'Elevage de l'Espece Bovine.

———— 1929 Fütterungslehre des Milchviehs. Die Quantitative Stoffwechselmessung und ihrer bisherigen Resultate beim Milchvieh. 246 pp., illus. Hannover.

RICHARDSON, H. B., AND E. H. MASON 1923 Clinical calorimetry. XXXIII. The effect of fasting in diabetes as compared with a diet designed to replace the foodstuffs oxidized during a fast. J. Biol. Chem., vol. 57, pp. 587–611.

'STUDENT' 1908 The probable error of a mean. Biometrika, Bd. 6, S. 1–25.

WIEGNER, G., AND A. GHONEIM 1930 Über die Formulierung der Futterwirkung. Ein Beitrag zur Theorie der Verwertung des Unterernährungs- und Produktionsfutters auf Grund von Neuen Fütterungsversuchen. Die Tierernährung, Bd. 2, S. 193–232.

A STUDY OF RACHITOGENIC DIETS COMPOSED OF PURIFIED FOOD MATERIALS[1]

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ONE FIGURE

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In order to produce rickets in rats it is not only necessary to have a diet which is deficient in the antirachitic vitamins but it must also be low in phosphorus. In the past such diets, for the greater part, have been composed of cereal grains such as yellow maize (Steenbock and Black, '25) or wheat (McCollum et al., '21; Sherman and Pappenheimer, '21). These grains owe their rachitogenic properties to the fact that they are low in phosphorus and a considerable portion of that present is not available for the rat (Bruce and Callow, '34). In addition it has been found necessary to decrease further the availability of the phosphorus by the addition of excess calcium carbonate. The advantages of a purified or of a so-called synthetic rachitogenic diet over the cereal diets are numerous. For example, it has been shown that the Steenbock-Black rachitogenic diet is deficient in vitamin A, protein and vitamin B complex (Kletzien et al., '32; O'Brien and Morgareidge, '38). It should be possible to correct these deficiencies with a synthetic diet. If the total amount of phosphorus is materially reduced it would be unnecessary to rely upon the unavailability of a portion of that present to produce rickets, and also it would allow for the production of severe rickets with greater regularity and possibly in a shorter time. Such a diet could also be reproduced with greater constancy than the cereal diets, because cereals vary in their content of both total and available phosphorus (Snook, '38), and, aside from its content of phosphorus, maize varies in its rachitogenic properties (Harris and Bunker, '33, '34). Furthermore, a diet composed of individually purified constituents would lend itself to the study of the various factors concerned in calcium and phosphorus metabolism much better than diets composed of natural food materials. The greatest difficulty in the preparation of a purified diet low in phosphorus has been to obtain readily a satisfactory and inexpensive protein. Casein which has been used so widely in synthetic diets is not suitable as it contains too much phosphorus. Several years ago Osborne, Mendel and Park ('23) described a purified rachitogenic diet containing either edestin or lactalbumin as the protein. However, possibly due to the proteins and also to the complete absence of vitamin A their diet was never used extensively. Recently we reported (Jones, '38) that in our search for a protein low in phosphorus we had found that blood-fibrin met the desired requirements. With fibrin as the purified protein a number of rachitogenic diets have been constructed and studied, some of which are presented at this time.

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[1] Presented before the biochemical section of the American Chemical Society, Milwaukee, September 5 to 9, 1938.

EXPERIMENTAL

Rats were used as test animals and were usually placed on the experimental diet when 25 days of age. Unless otherwise stated, 21 days later they were bled under ether anesthesia and the bloods from several rats (usually three) were united and the sera analyzed for calcium by the method of Clark and Collip ('25) and for inorganic phosphorus on the calcium-free filtrate by the method of Gunther and Greenberg ('29). The right femur was removed from each animal for ash analysis and the wrist bones were taken for examination by the line-test technic.

The composition of a number of the experimental diets and the phosphorus content of each are given in table 1. Salt mixture no. 5, which was used in several of the diets, is composed of the following ingredients expressed in relative parts: NaCl 1, $CaCO_3$ 3, KCl 1, $MgSO_4$ (anhydrous) 0.2, ferric citrate 0.1, and KI 0.004. When fed at a level of 5.3% of the ration this salt mixture furnished 1% NaCl and 3% calcium carbonate, which is the same as that in the Steenbock and Black ('25) and McCollum et al. ('21) rachitogenic diets. Diet 570 which has proved very successful is composed of alcohol-extracted fibrin 18%, brewers' yeast 5, salt mixture no. 5, 5.3, agar 2, carotene solution (Mead Johnson and Co.) 0.1 and dextrinized starch 69.6. The starch was dextrinized in the laboratory by autoclaving. It is necessary that the starch contain not more than traces of phosphorus. In diet 573 the dextrin was replaced by sucrose. In most cases in which sucrose comprised a large part of the ration the rats consumed insufficient food for the first 2 or 3 days to maintain their weight. This apparent reduction of appetite was of short duration, and with an increase in food consumption

TABLE 1

Percentage composition of several purified rachitogenic diets

Diet no.	570	573	610	576	611	612	618	623
Fibrin	18	18	18	18	18		18	18
Yeast	5	5		5			5	5
Salt no. 5	5.3	5.3	5.3	5.3	5.3	5.3	5.3	
Agar	2	2						
Carotene solution	0.1	0.1	0.1	0.1	0.1	0.1		
Dextrin	69.6						69.7	71
Sucrose		69.6	73.6	71.6	73.04	78.8		
Liver extract 343			1.0		1.0	1.0		
Thiamine			20[1]		20[1]			
Cellulose			2.0		2.0	2.0		
Casein						12		
Yeast concentrate						0.8		
K_2HPO_4					0.56			
Alfalfa							2	2
NaCl								1
$CaCO_3$								3
Total phosphorus	0.08	0.08	0.02	0.08	0.12	0.14	0.10	0.10

[1] Micrograms per day.

the animals showed considerable gain in weight. Diets 570 and 573 contain about 0.08% phosphorus and both produced severe rickets in 3 weeks or less.

The greater part of the phosphorus of these two diets is supplied by the yeast. Likewise, aside from the 0.1% carotene solution (and in some cases crystalline carotene was used) the only unpurified materials in diet 573 are the yeast and agar. Consequently in diet 610 (table 1) a higher degree of purity was obtained and the amount of phosphorus was simultaneously reduced by replacing the agar with an equal amount of regenerated cellulose[2] and supplying the B factors with 1% Lilly Liver Extract 343 and 20 micrograms of thiamine[3] daily. By so doing the phosphorus of the diet was reduced to 0.02%. This diet also produced marked rickets in a short time. In figure 1 are shown sections of a wrist bone from one animal on each of these three diets taken at the end of 3 weeks and prepared with silver nitrate in the usual manner. As can be seen the rachitic condition is very marked and the margins between the calcified and uncalcified areas are straight and regular. There is a complete lack of the so-called 'cupping' or extension of the calcified area along the margin of the bones. In some cases the degree of rickets was not as marked as that illustrated, but without exception well-defined rickets was obtained. Omission of the roughage as in diet 576 had no effect on the severity of the resulting rickets.

Having shown that it is possible to produce severe rickets on a highly purified diet, indicating that the only factors concerned with the production of rickets are vitamin D and the amount and availability of phosphorus, we next turned our attention to the effect of adding phosphorus to the diet on the development of rickets. When the phosphorus content of diet 610 was increased to 0.12% by the addition of 0.56% dipotassium phosphate (diet 611, table 1) the degree of rickets produced was less as indicated by an extension of the calcified area along the margin of the bone or the 'cupping' referred to above (fig. 1).

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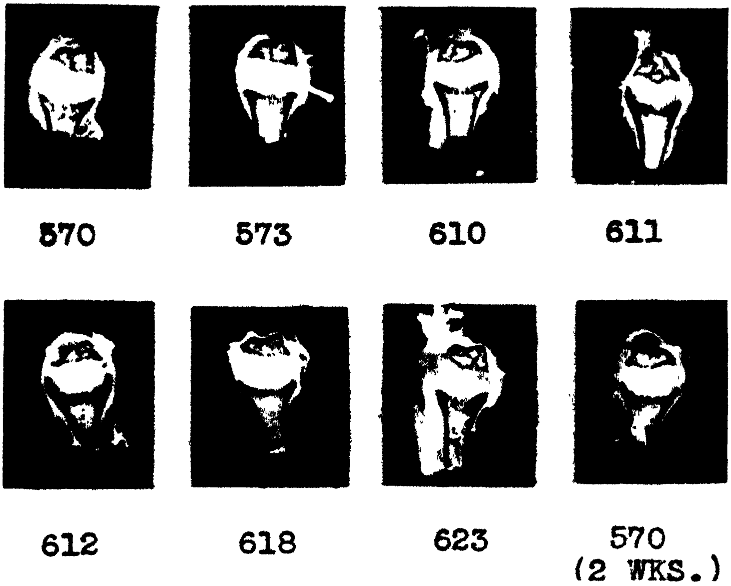
[2] Kindly sent to the author by Dr. C. M. McCay of Cornell University.

[3] Furnished by Merck and Company, Inc., Rahway, N. J.

A short time ago Harris and Bunker ('37) reported that they were unable to obtain rickets in rats on a diet containing casein even though the casein had been partially dephosphorized. Their diet, however, still contained about 0.2% phosphorus which, judging from our results, would be too high to allow for the production of rickets. Consequently an effort was made to reduce the phosphorus in a casein-containing diet considerably below this level. No steps were taken to remove the phosphorus chemically combined as part of the casein molecule, but purified casein[4] was fed at a level of 12% as indicated in diet 612 (table 1). The vitamin B factors were supplied with 1% liver extract and 0.8% Harris yeast concentrate. Such a diet contains about 0.14% phosphorus



570 573 610 611

612 618 623 570 (2 WKS.)

Fig. 1 Production of rickets on various diets. Numbers refer to diets described in text.

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[4] Supplied by Smith, Kline and French, Inc., Philadelphia, Pa.

and very definite rickets was produced. However, as shown in figure 1 there was some 'cupping' and the results were very similar to those obtained on diet 611 which had approximately the same amount of phosphorus. Similar results have been obtained with several different diets in which casein has been used as the protein. It thus appears that aside from its content of phosphorus casein has no special antirachitic properties. Under the conditions prevailing in this laboratory a rachitic picture quite like that resulting from feeding diets 611 and 612 has been frequently obtained on the Steenbock-Black diet ('25). From this it would seem there is probably from 0.10% to 0.15% of available phosphorus in this ration.

In an attempt to construct as inexpensive and simple a diet as possible the vitamin A and roughage of diet 570 were supplied by 2% of alfalfa, as shown in diet 618 (table 1). The phosphorus content of this diet is approximately 0.1% and very good rickets was obtained in 3 weeks (fig. 1). It was also found possible to reduce the fibrin to 12% without influencing the results. As the salt mixture used in these diets is somewhat more complex than that used by either Steenbock or McCollum, diet 618 was modified to contain 1% sodium chloride and 3% calcium carbonate in place of salt mixture no. 5, as shown in diet 623. Again a marked degree of rickets was produced (fig. 1).

In doing routine work it is desirable to shorten the length of each procedure as much as possible. In the last photograph of figure 1 is shown the results of feeding diet 570 for only 2 weeks instead of 3 weeks. It is obvious that on this diet well-advanced rickets can be obtained in no more than 14 days and possibly less. Recently Schneider and Steenbock ('38) have reported a diet low in phosphorus on which they were able to produce rickets in rats in a period of 2 weeks. As their diet contained 18% cooked egg white and 4% Vitab it cannot be classed as a purified diet. Furthermore, it is rather costly and somewhat laborious to prepare.

In table 2 are given the gains in weight of the animals, the ash content of the femurs and the level of calcium and inorganic phosphorus of the sera. The results are given as the averages for each group. For the most part, the rats gained about 25 gm. in weight during the experimental period of 3 weeks. When liver extract and thiamine replaced yeast as in diets 610 and 611 the gains were considerably less. Indications are that some of the B factors were not being supplied in sufficient quantity. In other experiments in which casein was the protein growth was below normal when the B complex was supplied as above and the diet was otherwise complete. Furthermore by increasing the liver extract good growth was obtained with fibrin but because it was desired to keep the phosphorus content of these rations as low as possible the level of liver extract was not increased in these experiments. There is little difference in the ash content of the femurs of the various groups except those on diets 611 and 612. These diets as stated above were higher in phosphorus and produced less severe rickets as shown by examination of the wrist bones. This increased calcification is reflected in both the amount and percentage of femur ash. In respect to the

TABLE 2

Effect of various rachitogenic diets on femur-ash and calcium and phosphorus of serum

DIET NO.	NUMBER OF ANIMALS	GAIN IN WEIGHT	FEMUR ASH		SERUM		
					Number of determinations	Ca[1] mg. per 100 cc.	P[1] mg. per 100 cc.
		gm.	*mg.*[1]	%[1]			
570	27	22.9	16.4	21.7	9	12.8	2.3
573	24	22.6	18.6	23.6	8	13.2	2.6
610	10	9.5	15.9	23.5	3	13.1	2.6
576	6	20.3	15.9	22.1	2	12.6	2.1
611	3	12.7	27.5	36.1	1	13.2	3.1
612	3	21.3	27.1	31.7	1	13.6	2.6
618	6	32.2	16.9	23.9	2	12.7	2.4
623	4	27.7	17.4	21.6	1	13.0	2.2

[1] Average of number of animals indicated.

serum, the inorganic phosphorus is considerably below normal and the calcium somewhat above. The phosphorus of the group of rats on diet 612 is slightly below that expected but of several groups on similar diets containing casein this was the lowest value for phosphorus obtained. As might be predicted the phosphorus of the serum of the rats given the extra phosphorus in their diet (diet 611) was a little above that of the others.

The above data demonstrate that it is possible to produce rickets in rats on a diet composed almost entirely of purified food substances if the phosphorus content is maintained at a low level. This furnishes additional evidence in favor of the view that cereals do not owe their rachitogenic properties to the presence of a specific anti-calcifying agent. Also it gives a better opportunity for a study of the factors leading to the development of rickets in the rat. Whether such diets can be used to advantage in the routine assay of materials for their vitamin D content remains to be determined.

SUMMARY AND CONCLUSIONS

A number of low-phosphorus diets rachitogenic for the rat and composed of purified food material have been described. Blood fibrin was used as the protein in most of the diets. A fair degree of rickets can be obtained with casein if fed at a level of not over 12%. Apparently the antirachitic activity of casein is due entirely to the large amount of phosphorus contained in this protein. In order to produce severe rickets in the rat on diets containing 3% $CaCO_3$ the available phosphorus must not be greater than approximately 0.1%.

LITERATURE CITED

BRUCE, H. M., AND R. K. CALLOW 1934 Cereals and rickets. The role of inositolhexaphosphoric acid. Biochem. J., vol. 28, p. 517

CLARK, E. P., AND J. B. COLLIP 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, p. 461.

GUNTHER, L., AND D. M. GREENBERG 1929 A note on the determination of the inorganic phosphate of the serum on the filtrate from calcium analysis. J. Biol. Chem., vol. 82, p. 551.

HARRIS, R. S., AND J. W. M. BUNKER 1933–1934 Variability in the corn component of a rachitogenic diet. J. Lab. Clin. Med., vol. 19, p. 390.

——— 1937 The antirachitic property of casein. J. Biol. Chem., vol. 119, p. xlv.

JONES, J. H. 1938 The use of fibrin in synthetic diets. J. Nutrition, vol. 15, p. 269.

KLETZIEN, S. W. F., V. M. TEMPLIN, H. STEENBOCK AND B. H. THOMAS 1932 Vitamin D and the conservation of calcium in the adult. I. J. Biol. Chem., vol. 97, p. 265.

MCCOLLUM, E. V., N. SIMMONDS, P. G. SHIPLEY AND E. A. PARK 1921 Studies on experimental rickets. VIII. The production of rickets by diets low in phosphorus and fat-soluble A. J. Biol. Chem., vol. 47, p. 507.

O'BRIEN, B., AND K. MORGAREIDGE 1938 Radiographic demonstration of protection by vitamin D against metaphyseal decalcification in adult rats on high calcium-low phosphorus diets. J. Nutrition, vol. 16, p. 91.

OSBORNE, T. B., L. B. MENDEL AND E. A. PARK 1923–1924 Experimental production of rickets with diets of purified food substances. Proc. Soc. Exp. Biol. and Med., vol. 21, p. 87.

SCHNEIDER, H., AND H. STEENBOCK 1938 Differences in response to vitamin D_3 of rats on cereal low phosphorus diets and synthetic low phosphorus diets. J. Biol. Chem., vol. 123, p. cv.

SHERMAN, H. C., AND A. M. PAPPENHEIMER 1921 Experimental rickets in rats. I. A diet producing rickets in white rats, and its prevention by the addition of an inorganic salt. J. Exp. Med., vol. 34, p. 189.

SNOOK, L. C. 1938 Cereal grains as a source of nutritionally useful phosphorus. Empire J. Exp. Agr., vol. 6, p. 20.

STEENBOCK, H., AND A. BLACK 1925 Fat-soluble vitamins. XXIII. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem., vol. 64, p. 263.

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SUPPLEMENT

PROCEEDINGS OF THE SIXTH ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

KING EDWARD HOTEL, TORONTO, APRIL 26, 1939

The sixth annual meeting of the American Institute of Nutrition was held in Toronto at the King Edward Hotel on April 26, 1939. Ninety-four members and 420 guests registered, but there were approximately 800 in attendance during the scientific sessions.

COUNCIL MEETING

The Council meeting was held at the King Edward Hotel Tuesday afternoon, April 25th, 2.00 P.M. All members were present. Formal actions of the Council are reported in the minutes of the general business session.

SCIENTIFIC SESSIONS

President E. V. McCollum presided at the first scientific session and at the business meeting. Vice-president T. M. Carpenter presided at the second scientific session. The scientific program was begun at 9.30 A.M., April 26th, and proceeded on schedule time. All the papers listed on the program were given.

The Symposium program held on the evening of April 26th under the chairmanship of President McCollum, was attended by over 800 persons. All of the papers had been very carefully prepared by the speakers and their presentations held the very close attention of the large audience. From the comments received it was evident that this program proved one of the most attractive features of the annual meeting.

BUSINESS SESSION

The business meeting convened at 11.30 A.M., April 26th, and was called to order by President McCollum. The reading of the minutes of the previous annual meeting was dispensed with since they had been published in the Journal.

In accordance with the by-law providing that members in good standing who have reached the age of 65 years shall become emeritus members, it was announced that Paul Roth has attained this rank of service.

The report of the treasurer was read by George R. Cowgill. The auditors, W. H. Sebrell and H. W. Titus, previously appointed by the President, reported that they had examined the treasurer's books and found them correct. It was moved by P. E. Howe and seconded by Helen S. Mitchell that the report of the treasurer be accepted. The motion carried.

The Council recommended to the members that the annual dues for 1939–1940 be $1.00 and the registration fee for the 1940 meeting be 50 cents. It was so moved by G. R. Cowgill and seconded by W. H. Sebrell. The motion carried.

The Council recommended that the following candidates be elected to membership in the American Institute of Nutrition:

Paul Gyorgy
J. S. Hughes
E. L. Sevringhaus
Elizabeth C. Robertson
Susan G. Smith
Elizabeth Neige Todhunter

A. H. Smith moved and H. D. Branion seconded that the secretary cast a unanimous vote for the election to membership of the candidates approved by the Council. The motion carried and the secretary declared the candidates duly elected members of the American Institute of Nutrition.

J. R. Murlin reported informally as Editor. The items mentioned in his report are covered in the formal report of the Editorial Board which is included later.

G. R. Cowgill reported for the committee appointed to draw up regulations for the administration of the Mead Johnson and Company Award for Research on the vitamin B complex. The secretary called attention to the public announcement of the principles governing the award as published in the Journal, vol. 16, p. 621, 1938, and enumerated certain other regulations which had been approved by the Council. On motion by L. C. Norris the principles outlined were approved. As a matter of record the detailed statement approved by the Council is included in these minutes.

The Mead Johnson and Company Award for Research on the Vitamin B Complex

G. R. Cowgill as chairman of the committee consisting of L. A. Maynard, C. A. Elvehjem, Icie Macy Hoobler, and A. G. Hogan, reported on the work of the committee in formulating the principles for the administration of the Mead Johnson and Company Award. (The report of this committee was circulated to the Council in November, 1938 and was approved by a mail vote. See attached sheets.) The secretary read the following statement based upon the above report, which had been transmitted to the Committee of Judges and which was published in part in the Journal (*16*, 621, 1938) and elsewhere as a public announcement:

Statement Regarding the Administration of the Mead Johnson and Company B Complex Award

In order to promote interest in researches dealing with the water-soluble B-complex vitamins, Mead Johnson and Company have established an annual award of $1000.00 to be given over a period of 5 years through the medium of the American Institute of Nutrition. The Institute, through an appropriate Committee of Judges selected from its membership, will name the recipient of this Award; the formal presentation of it will be made as part of the program of the annual meeting held each spring.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st, the most meritorious scientific report dealing with the field of the B-complex vitamins. While the award will be given primarily for publication of specific papers, the judges will be given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

A Committee of Judges consisting of five members of the Institute will be appointed annually by the President to recommend to the Council a recipient for the award. To assist this committee in its deliberations nominations will be solicited by the Secretary of the Institute by means of public announcements and by a note in the annual letter to members. Such nominations may be sent to the Secretary at any time for transmissal to the Committee of Judges, but for a nomination to be considered for the award to be bestowed at any given spring meeting of the Institute, however, it must be received by the Secretary not later than January 15th. The nomination should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination. In addition to the investigation of the merit of nominations thus made it will be the duty of the Committee to search the literature on its own account in arriving at its selection of a recipent for the Award. The personnel of the Committee will be kept confidential until its deliberations are finished.

The name of the nominee selected by the Committee of Judges should be sent to the Secretary of the Institute as soon as possible after January 15th. This nomination will then be submitted to the Council for ratification and thereafter the nominee will be notified in order that he may make plans for being present at the annual meeting. The name of the nominee will be kept confidential by all concerned until the announcement is made at the annual dinner, at which time the Award will be bestowed by the President of the Institute. The recipient will be expected to make a brief response.

It is a very definite desire of both the donor and the Council that the award be made annually. In accepting the award, the Council specified that the field covered should be broad enough so that there should be no question about finding a meritorious contribution and recipient annually. It has been understood with the donor, however, that the Institute will not be expected to make the Award for definitely mediocre work. Thus, the Council is prepared to consider the withholding of the Award in any given year if it is the carefully considered judgment of the Committee of Judges that this should be done.

In addition to the purse provided by Mead Johnson and Company the recipient will be presented with a simple scroll as a memento of the occasion, the cost to be defrayed by the Institute.

This statement was approved by the Council on the motion of G. R. Cowgill, seconded by P. E. Howe, and it was voted to recommend its adoption by the Institute as a basis for future awards. As a matter of record, President McCollum reported the appointment of the following Committee of Judges for the 1938–1939 award: G. C. King, chairman, W. C. Rose, H. B. Lewis, Helen T. Parsons, F. F. Tisdall.

The Secretary announced that in addition to the purse of $1000 Mead Johnson and Company had agreed to provide a sum of $100 annually for the traveling expenses of the recipient.

The Council recommended that the Institute hold its 1940 meeting on the day prior to the meetings of the Federation. This recommendation was adopted on the motion of A. G. Hogan, seconded by E. M. Nelson.

President McCollum appointed the following nominating committee for 1939–1940: W. C. Russell, chairman, P. L. Day, Agnes Fay Morgan, F. F. Tisdall, H. W. Titus.

The Secretary presented a communication from R. G. Harrison, Chairman of the National Research Council, relative to the Sixth Pacific Science Congress to be held in California July 24 to August 12, 1939. By request of the secretary, Agnes Fay Morgan made a statement regarding the purpose and program of this Congress. It was moved and carried that the incoming president be requested to appoint certain members of the Institute as its representatives to this Congress, in accordance with the request in the letter from Doctor Harrison.

President McCollum asked the tellers, E. M. Nelson, chairman, and A. G. Hogan, to report on the count of the ballots for the election of officers for 1939–1940. The results were as follows: President, H. C. Sherman; Vice-President, T. M. Carpenter; Secretary, L. A. Maynard; Treasurer, G. R Cowgill; Councilor, A. H. Smith; Associate Editors, Icie Macy Hoobler, H. A. Mattill, H. H. Mitchell.

It was moved by Helen S. Mitchell and seconded by L. C. Norris that the secretary be instructed to extend for the members of the American Institute of Nutrition and their guests their sincere thanks to the local committee and the management of the King Edward Hotel for the excellent arrangements provided for the meeting and for the many courtesies extended.

LUNCHEON

The noon luncheon in the Alexandra Room of the King Edward Hotel was attended by 217 members and guests.

DINNER

The subscription dinner held in the Alexandra Room of the King Edward Hotel was attended by 286 members and guests.

The Mead Johnson and Company Award for Research on the Vitamin B Complex for 1939 was presented by President McCollum to Conrad Arnold Elvehjem, Professor of Biochemistry, University of Wisconsin. In addition to the purse of $1000 provided by the Mead Johnson Company, Doctor Elvehjem was presented with a scroll which stated the basis of the Award in the following words "In recognition of his outstanding research accomplishments in relation to the vitamin B complex, particularly the importance of nicotinic acid and related compounds in nutrition."

In making the presentation President McCollum paid tribute to the generosity of the Mead Johnson Company in providing the Award and expressed the belief that it would serve an important purpose in stimulating research in nutrition. In response Doctor Elvehjem expressed his gratitude for the Award, described briefly the work which led up to his discoveries and paid tribute to his teachers who had inspired him and to his colleagues who had been associated with him in the work.

Sir John Boyd Orr, director of the Rowett Institute, Aberdeen, Scotland, a special honor guest at the dinner, was called upon and spoke briefly regarding the importance of nutrition research and brought greetings from the nutrition workers in the British Empire.

EDITORIAL BOARD MEETING

The Editorial Board met at the King Edward Hotel at the dinner hour on April 27, 1939. Present were J. R. Murlin, A. H. Smith, Grace MacLeod, J. B. Brown, E. M. Nelson, L. H. Newburgh, H. A. Mattill, G. R. Cowgill and A. G. Hogan. After considerable discussion regarding the acceptance of papers from foreign contributors it was voted that a limited number of such papers of outstanding merit may be accepted for publication beginning with volume 18. The number accepted should be left to the discretion of the editor depending on the pressure of publication from contributors from the U. S. and Canada. As a guiding figure it was suggested, though not formally adopted, that not more than 5% of the total number of papers appearing in any volume should be from foreign contributors.

The editor presented again the possible necessity of publishing three volumes per year in the near future. The editor had asked the opinion of The Wistar Institute on a proposal that, if they could see their way clear to publish three volumes a year, the subscription rate to members of the American Institute of Nutrition should be $3.50 per volume; to all others $5.00 per volume. This would represent a discount of 30% instead of the 20% now prevailing for members. Because of the untimely death of Dr. Alfred G. Stengel, who has been acting as director of The Wistar Institute since the death of Doctor Greenman, it was not possible for the Wistar to give a definite answer on this proposal before the Toronto meeting.

A midway position would be to extend the number of pages in each volume to possibly 720 pages instead of 600 as at present with a proportional increase in subscription price. No decision on this matter was reached, but it will obviously come up again at the meeting of the Board next year.

The following data were presented to the Board regarding the number of papers published in the Journal since it last met:

Number accepted	103
Number rejected	50 = 33%
Withdrawn (did not wish to condense)	1
	154

In order to indicate how the new policy adopted last year, of requesting all contributors to condense their material as much as possible, had worked out, the following figures were given:

	Average pages per article
May, 1938	16
June	13
July	14.5
August	13
September	10
	Av. 13.3

	Average pages per article	
October	9.8	
November	13	
December	11	
January, 1939	9	
February	12	
March	10.4	
April	13	
		Av. 11.1
Average for year	12.2	

		Number of articles per volume
Volume	13	48
	14	51
	15	49
	16	54
	17	55

The new rule obviously could not go into effect immediately after the meeting in Baltimore because papers had already been accepted for issues up to and including September, 1938. Consequently the figures up to this point represent the data previous to application of the rule. From October to April, 1939, they represent data under the new rule. It is evident that there has been a gain in the way of reducing the length of papers, and, it is believed, without injury to the presentation of results. It is evident also that there has been a steady gain in the number of papers published per volume since 1937.

It was the consensus of opinion of those present that continued effort to induce authors to be saving of Journal space as much as possible should be continued.

RESIGNATION OF EDITOR

At this point Doctor Murlin submitted his resignation as editor of the Journal of Nutrition to become effective July 1, 1939. He stated that he was moved to take this action for two reasons. First, because of the by-law prohibiting holding of other offices after the age of 65 and secondly, because he felt that he had served sufficiently long.

A motion was offered by Doctor Brown and seconded by Doctor Hogan that the resignation be accepted with regret but at the same time with appreciation of Doctor Murlin's services.

A procedure for the election of a successor was then adopted. The Board obviously was unprepared to proceed to an election at once. It was agreed that each member of the Board present should canvass active members of the Institute of Nutrition while at the Toronto meeting and then submit to Doctor Murlin by May 8th the names of promising candidates. It was agreed also to have an informal discussion of names suggested at an adjourned meeting on the morning of April 28th.

At this meeting Doctors Chambers and Shaffer of the Editorial Board also were present. Doctor Murlin offered the services of his office as a clearing house for the ballots which would obviously have to be taken by mail. This offer being agreeable, he requested that all members of the Editorial Board submit any additional nominations to him not later than May 8th in order that the election might be completed by July 1st. He agreed to submit as soon as possible thereafter the full list of names to each member of the Board as now constituted for an expression of their first, second and third choice. The first choice would be given a weight of 3, the second a weight of 2 and the third choice a weight of 1. The three candidates having the highest total rating would then be submitted to the Board for a second preferential ballot. If no candidate received a majority vote, the one with the lowest total rating would be dropped and a third ballot taken on the other two.

Respectfully submitted,

L. A. Maynard
American Institute of Nutrition

ABSTRACTS OF PAPERS

Relationship between carotene, blindness due to constriction of the optic nerve, papillary edema and nyctalopia in calves. L. A. Moore (introduced by C. A. Hoppert), Dairy Department, Michigan State College, East Lansing.

For the past 20 years various investigators have noted a peculiar type of blindness in calves associated with rations where poor quality or no roughage was fed. Previous work has shown that the blindness is due to a constriction of the optic nerve where it passes through the optic foramen caused by a stenosis of the bony canal. Further work with calves has shown that the blindness is preceded by nyctalopia and ophthalmoscopic observations revealed papillary edema and bleaching of the Tapetum Lucidum. Holstein calves placed on the blindness producing ration and fed crystalline carotene dissolved in cottonseed oil in quantities sufficient to keep the blood plasma carotene level well above a lower limit of 0.13 micrograms per milliliter did not develop these eye changes. Vitamin A deficiency is therefore the causative factor.

In mature cows nyctalopia and papillary edema develop but blindness due to constriction of the optic nerve does not develop due to the fact that the bony optic canal has already been formed. In calves blindness due to constriction of the optic nerve and papillary edema are associated while nyctalopia is a separate process but due to the same deficiency.

Papillary edema is a cardinal sign of increased intracranial pressure. Vitamin A may therefore be necessary for the regulation of this pressure. Increased intracranial pressure may also account for certain nervous manifestations of vitamin A deficiency.

Vitamin A requirements of adults. L. E. Booher, E. C. Callison (by invitation), E. M. Hewston (by invitation), and R. Loughlin (by invitation), Bureau of Home Economics, U. S. D. A., Washington, D. C.

The vitamin A storage of normal well-nourished adults is, on the average, sufficient to prevent detectable signs of nutritional night-blindness for a period of about 1 month. The daily intake of vitamin A which just suffices to maintain normal dark adaptation as determined for five adult subjects varied between the limits of 25 and 55 international units per kilogram of body weight. The daily intake of carotene in cottonseed oil which just suffices for maintenance of normal dark adaptation of adults as measured on ten subjects varied between the limits of 43 and 103 international units per kilogram of body weight. In comparison with cod liver oil, the vitamin A potency of carotene in cottonseed oil was only about 50 to 60% utilized. Further studies have been made in this laboratory to determine the effect of different levels of dietary fat and vitamin B_1 on the utilization of carotene in cottonseed oil for the maintenance of normal dark adaptation of adults. These results do not offer the complete solution to the problem of carotene utilization by adults. Investigation of the total carotene content of peas and of spinach samples in conjunction with simultaneous determination of the international unit values and the minimum vitamin A requirements of adults referred to these same samples of vegetables will be discussed.

Experimental evidence of human riboflavin deficiency. W. H. Sebrell and R. E. Butler (by invitation), National Institute of Health, Washington, D. C.

Eighteen women were given a ration of cornmeal, cowpeas, lard, casein, flour, white bread, sirup, calcium carbonate, tomato juice, cod liver oil, sirup of iodide of iron, ascorbic acid and thiamin chloride. Eleven of the eighteen developed lesions of the lips, angles of the mouth, and seborrheic changes on the nose or ears in from 94 to 185 days. One of the remaining seven started a daily preventive dose of synthetic riboflavin on the one hundred and twenty-ninth day and three on the one hundred and thirty-ninth day. No lesions appeared by the two hundred and twenty-second day.

Six of the women with lesions were given nicotinic acid without benefit. Five then rapidly healed and one slowly improved after the administration of synthetic riboflavin. Treatment was discontinued as soon as healing was complete, and by the two hundred and fourth day lesions had reappeared in three.

The remaining five cases responded to treatment with synthetic riboflavin without nicotinic acid. Treatment was discontinued as soon as healing was complete, and by the one hundred and eighty-sixth day the lesions had reappeared in two.

In the five cases with recurrences the first lesions were completely healed after a total dosage of 11.41 to 34.28 mg. of synthetic riboflavin and there was an interval of 51 to 90 days between the appearance of lesions.

No significant variation in food consumption or body weight was observed during the administration of riboflavin.

Nicotinic acid: its distribution, function and relation to the other members of the B complex. C. A. Elvehjem, Harry A. Waisman (by invitation), and A. E. Axelrod (by invitation), Department of Biochemistry, University of Wisconsin. Madison.

The nicotinic acid activity of a number of foods has been determined by comparing the response made by dogs suffering from black tongue to standard amounts of nicotinic acid and to different levels of the foods tested. Typical results are as follows: liver 1.0 to 1.4, kidney 0.8 to 1.0, lean muscle tissue 0.4 to 0.7, brain 0.4 to 0.5, yeast 0.4 to 0.5, heart 0.2 to 0.3 mg. per gram dry tissue. Wheat germ, milk (fresh and skim milk powder) and dried grass showed a lower potency.

In attempts to determine the physiological activity of nicotinic acid, changes in the cozymase content of tissues from dogs and pigs suffering from nicotinic acid deficiency were studied. The cozymase was determined by the Euler-Myrbäck yeast fermentation method. No consistent variation from the values for normal animals could be demonstrated in the blood, kidney or brain, but the muscle and liver showed very significant decreases. The nicotinic acid necessary to account for the cozymase content of normal tissues agrees in a general way with the amount found in these tissues by animal assay.

Further studies on the differentiation of nicotinic acid from other members of the B complex and the relation of these factors to the optimum utilization of nicotinic acid will also be discussed.

Field studies on the diets of pellagrins. T. D. Spies, J. M. Grant (by invitation) and Martha Hargrove (by invitation), General Hospital, Cincinnati; Hillman Hospital, Birmingham.

The present communication is a report of field studies in which the dietaries of seventy pellagrins (fifty adults and twenty children) were investigated and analyzed. The purpose of these studies was to ascertain the amount and kind of food actually eaten by these persons in the home; to determine existing deficiency states and the actual degree of deficiency; and to apply methods of correction. The diets of these people not only are inadequate in caloric value, but their protein content is low and its biological quality poor. The fat content is very low. While the actual carbohydrate content of these diets is not high, the proportion of calories derived from carbohydrates to those derived from fats is extremely high, a factor which probably results in an increased requirement for vitamin B_1. Aside from the pellagra-preventive factor, vitamin B_1 and riboflavin are probably the outstanding vitamin deficiencies. The calcium, phosphorus and iron content of the pellagrin's diet is low.

The addition of nicotinic acid in adequate amounts to such a diet will protect against certain symptoms, but oftentimes vitamin B_1 and flavin deficiency will develop. Persons who exist on these diets, a representative one of which is shown in the slide, eventually develop pellagra; many of them develop beriberi; and certain of them develop flavin deficiency.

The addition of synthetic nicotinic acid, synthetic riboflavin, and synthetic vitamin B_1 in adequate amounts as daily supplements to the diet eaten by the endemic pellagrins greatly increases their strength, feeling of well-being and ability to work. If nicotinic acid is omitted, the persons are prone to have relapses of the alimentary and central nervous system manifestations of pellagra. If the vitamin B_1 is omitted, they are prone to have relapse of beriberi. If flavin is omitted, they are prone to have recurrences of the manifestations of flavin deficiency

Factors affecting the excretion and requirement of vitamin B_1 in small children. E. M. Knott (by invitation) and F. W. Schlutz, Department of Pediatrics, The University of Chicago.

In vitamin B_1 balance studies, based on 5-day metabolism periods, excretion of the vitamin was about equally divided between urine and feces for preschool children, whereas infants excreted but little in their feces. Although individual differences were encountered, retentions of thiamin were somewhat related to carbohydrate intake and were more definitely influenced by the level of thiamin intake.

When supplements of either thiamin or wheat germ were given to six children receiving basic diets differing in their content of whole grain cereals, the added vitamin usually increased urinary excretion, although individual differences were encountered both in ability to absorb the vitamin and in the amount appearing in the urine. In five out of twelve periods the wheat germ was absorbed slightly less than was the thiamin. In three children poorer absorption was observed when whole grain cereals were included in the basic diet. The more constipated children had a higher fecal excretion presumably due to synthesis of the vitamin since administration of sufficient mineral oil to correct the constipation caused a

50% decrease in the amount of vitamin B_1 in the feces. That the amount of roughage in a child's diet had a definite effect on his ability to digest and absorb vitamin was further illustrated when a milk diet shifted a child's excretion pattern to the infantile type of low fecal and high urinary elimination.

Nutritional cytopenia (vitamin M deficiency) in monkeys receiving the Goldberger diet supplemented with nicotinic acid. P. L. Day, W. J. Darby (by invitation) and W. C. Langston (by invitation), School of Medicine, University of Arkansas, Little Rock.

Young rhesus monkeys were given a diet essentially the same as the Goldberger blacktongue-producing diet no. 268.[1] They developed the syndrome characterized by marked anemia and leukopenia which has been described in monkeys receiving our diet 600.[2] Supplementing the Goldberger diet with 10 or 20 mg. of nicotinic acid daily, or with 20 or 40 mg. of nicotinic acid amide daily did not prevent the cytopenia. Two samples of nicotinic acid were used, one of which had previously been shown to be effective in curing blacktongue in dogs. The simultaneous administration of riboflavin, thiamin chloride and ascorbic acid failed to prevent the cytopenia. However, the diet supplemented with 2 gm. daily of Lilly's liver extract has promoted good growth and maintained a normal blood picture. Likewise, the feeding of liver extract to an animal with profound anemia and leukopenia was followed by a dramatic reticulocyte response and ultimate recovery. We interpret these data to indicate that this blacktongue-producing diet supplemented with nicotinic acid, ascorbic acid, riboflavin and thiamin chloride is deficient in some factor essential to the nutrition of the monkey. We have previously termed this factor vitamin M.

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[1] Goldberger, J., and W. H. Sebrell, Pub. Health Rep., U. S. P. H. S., vol. 45, p. 3064 (1930).

[2] Langston, W. C., W. J. Darby, C. F. Shukers and Paul L. Day, J. Exp. Med., vol. 68, p. 923 (1938).

The lability of the basal metabolism in growing rats. T. S. Hamilton, Animal Nutrition Division, University of Illinois, Urbana.

The basal metabolic rates of nearly 300 growing rats, under rigidly controlled conditions, were determined. The average basal metabolism of growing male rats fed well-balanced diets was 773 calories per 24 hours per square meter of body surface. Variations between 600 and 1000 calories were encountered, often in the same rat in less than 4 weeks' time. Using 1) pair mates gaining at different rates on the same amounts of different diets, 2) pair mates gaining at the same rates on different amounts of different diets, and 3) pair mates gaining at different rates on different amounts of the same diet, it was found that the chief factor affecting the B.M.R. of growing rats was the previous (21 days) caloric intake. The correlation between previous caloric intakes and basal metabolic rates, both expressed in calories per square meter per day was, for 228 rats, +0.706. The following factors were eliminated as factors of any great importance in affecting the B.M.R. of the rats studied: 1) the protein content of the diet, 2) the total amount of protein consumed, 3) rate of growth (although no rat studied had been losing weight), 4) composition of rat, 5) activity of rat and 6) temperature of environment.

Chemistry of the essential fatty acids. G. O. Burr, J. B. Brown, J. P. Kass (by invitation), and W. Lunberg (by invitation), Department of Botany, University of Minnesota, Minneapolis.

During the past year an intensive study has been made by a group here of the chemistry of the fatty acids which will cure fat-deficient rats. With natural oils, fatty acid concentrates and pure fatty acids comparative studies have been made. Acids from several sources have been used in the search for evidence of isomeric forms of special value. Especial emphasis has been laid on linoleic acid because of the many statements about natural isomeric forms. Our evidence indicates that there is only one widely distributed linoleic acid in seeds. Isomeric forms have been synthesized and they have all been inactive nutritionally. The isomer found in butter is probably less active than the common plant acid. Furthermore, linoleic acid is more useful in curing and preventing fat deficency than either linolenic, arachidonic or the other higher unsaturated acids in fish oils.

Methods of analysis have been carefully checked, and it has been found that the thiocyanogen calculation for linoleic acid is not a reliable measure of the curative value of all oils.

Some observations on the effects of low levels of dietary protein. A. U. Orten (introduced by A. H. Smith), Laboratory of Physiological Chemistry, Yale University, New Haven; and Department of Physiological Chemistry, Wayne University College of Medicine, Detroit, Michigan.

The effects of a chronic protein deficiency on both young and adult rats have been studied. The animals were fed a synthetic diet low in protein of excellent quality (lactalbumin 3.5 or 5%) with an abundance of all known essential factors other than protein.

The adult female rats given a low-protein diet remained in good condition for more than 4 months. No gross abnormalities were noted other than a striking tendency toward obesity. Further lowering of the protein caused a loss of weight and cessation of the oestrus cycle.

At 100 days of age, animals fed the low-protein diet from weaning were in good condition and resembled 30-day-old normal rats. However, they exhibited an hypochromic anemia characterized by a nearly normal erythrocyte count, a low hemoglobin, and an increased reticulocyte count. They showed slight, if any, evidence of sexual development. At autopsy the ovaries and reproductive tract were found to be infantile. Treatment with gonadotropic hormone and pituitary assays indicated that the pituitary rather than the ovary was at fault. Fatty livers and stomach ulcers were also observed, particularly in the adult animals. Findings in calorie, age and weight control groups indicated that the foregoing abnormalities are referable to the low protein content of the diet rather than to a diminished intake of calories or of other factors.

The effect of heat and hydration on the nutritive value of soy bean proteins. H. T. Parsons, A. L. Marlatt (by invitation), D. Johnson (by invitation), and C. Gray (by invitation), Nutrition Laboratory, Department of Home Economics, University of Wisconsin, Madison.

Gradated increases in the degree of heat treatment of soy beans resulted in increases in the nutritive value of the protein, for rats, up to short periods of autoclaving. Beyond this point, however, increases in the time or the temperature of autoclaving resulted in progressive losses. The poor results were not attributable to lack of the B complex in the ration as 'Vitab' produced no improvement; all rations were supplemented with thiamine. The addition of lysine was also without beneficial effect. Soaking the beans before boiling or autoclaving had a detrimental effect.

The effects of roasting depended upon how this was carried out; roasted, ground soy beans were practically no better than raw, whereas whole soy beans roasted at 150° for 30 minutes were more efficient than after some degrees of autoclaving. This pronounced effect of some relatively slight differences in hydration during heating and the tendency for prolonged autoclaving at high temperatures to be detrimental may explain the occurrence of discrepancies in the results of previous studies from various laboratories on the effect of heat on soy bean protein. The variety of soy bean, on the other hand, probably has had little significance in such differences since the comparative effects of various heat treatments were very similar for five varieties of dry mature soy beans and for six green garden varieties.

The amino acid composition of the proteins of cow and human milk. E. F. Beach (introduced by Icie Macy Hoobler), O. Hoffman (by invitation), and S. S. Bernstein (by invitation), Research Laboratory, Children's Fund of Michigan, Detroit, Michigan.

A fundamental consideration in determining the nutritive value of proteins is their content of various amino acids. We have prepared two protein fractions from samples of cow and human milks; casein by isoelectric precipitation and soluble-protein by precipitation from the whey at a concentration of 8% trichloracetic acid. These fractions were analyzed for basic amino acids by the Block method and for tyrosine and tryptophane by the method of Folin and Marenzi. The distribution of total nitrogen between casein, 'soluble-protein' and non-protein nitrogen in the various samples of milk was determined.

Cow's milk contained 494 mg. per cent protein nitrogen of which 405 were casein and 89 were 'soluble-protein' nitrogen. Human milk contained 126 mg. per cent protein nitrogen of which 49 were casein and 77 were 'soluble-protein.' The amino acid distribution in cow's milk was: histidine, 55.6; arginine, 114.5; lysine, 207; tyrosine, 192.2 and tryptophane, 39.6 mg. per cent. In human milk the distribution was: histidine, 10.5; arginine, 31.4; lysine 42.4; tyrosine, 44.1; and tryptophane, 11.5 mg. per cent.

Although cow's milk has approximately four times the protein concentration of human milk, the amino acid distribution in the proteins is similar. On the basis of five amino acids, the total protein of the two milks have equal nutritive value. The methionine and cystine distributions will be reported.

The purine metabolism of the Dalmatian dog. H. C. Trimble, Biochemical Laboratory, Harvard Medical School, Boston.

Adult male Dalmatian dogs on a purine-free diet excrete uric acid at the rate of 35 mg. per kilogram per day. Females averaged about 5 mg. less. Growing puppies excrete uric acid from birth, even before the spots appear. During the period of active growth the excretion rate of puppies is 50 to 70 mg. per kilogram per day. In adults increasing the carbohydrate, fat or protein content of the diet has not produced significant increases of uric acid output. In adults the output of allantoin has been observed to range from 20 to 50 mg. per kilogram per day. Increases of carbohydrate or fat have not enlarged allantoin output while increases of protein have been accompanied by very definite enlargements. Intravenous injections of uric acid have had very little effect upon allantoin output and the uric acid has been recovered quantitatively. These findings favor the conclusion that uric acid and allantoin are not completely interconnected with respect to their metabolism in the Dalmatian dogs. Supporting this conclusion are observations (preliminary reports of which have been made) upon the effects of total absence of the liver and upon the presence of uricase in the liver of Dalmatians.

An improved synthetic ration for the chick. A. G. Hogan and L. R. Richardson (by invitation), Department of Agricultural Chemistry, University of Missouri, Columbia.

Our first synthetic rations became undependable after a time and it developed that the liver extracts, obtained from various commercial sources, were of uneven usefulness for our purpose. We then began preparing them in the laboratory and these extracts were equal or superior to the best that had been previously available. Growth is normal and leg deformities are infrequent. Apparently the incidence of these abnormalities is independent of the amount of manganese in the ration.

In the original procedure liver was first extracted with strong alcohol and the extract adsorbed with fuller's earth. On a dry matter basis the filtrate made up 6% of the ration and the adsorbate was supplied in an equivalent amount. The liver residue was then extracted with water and this extract made up 6% of the ration. This extract is the most important and may be the only fraction that is essential. The other vitamin carriers are 8% of soy bean oil and 2% of cod liver oil. The liver residue still contains a substance of importance in chick nutrition and when the ration contains 10% of this material the rate of growth is remarkably rapid.

A chronic congenital ataxia and osteodystrophy in chicks due to manganese deficiency. L. C. Norris and C. D. Caskey (by invitation), Department of Poultry Husbandry, Cornell University, Ithaca, N. Y.

In studies on the role of manganese in the reproduction of the domestic fowl an ataxia was observed in the chicks of the hens fed a diet containing approximately 6 μg. of manganese per gram but no ataxia was observed in the chicks of hens fed higher levels. The ataxia accompanied the osteodystrophy which usually develops in the chicks of hens on this dietary regime. The chief symptoms of the ataxia were incoordination of head movements and gait; those of the osteodystrophy shortening of the bones of the legs and wings. Some of the head movements of the ataxic chicks were similar to those in polyneuritis.

Many of the ataxic chicks survived when fed a diet adequate in manganese but complete recovery was not made from either the ataxia or the osteodystrophy.

Normal chicks were hatched from the eggs of the ataxic pullets reared to maturity upon being artificially inseminated with the semen of ataxic cockerels. Therefore, the chronic ataxia and osteodystrophy in the pullets did not affect their ability to metabolize manganese and to produce normal offspring.

The ataxia was not cured by injection of manganese or thiamin or both manganese and thiamin. In a preliminary histological examination of the brains of ataxic chicks a marked reduction in the number of Purkinje cells of the cerebellum was observed. If this observation is confirmed, it offers an explanation of the inability to cure the ataxia.

The copper metabolism of young women. R. M. Leverton (introduced by L. J. Roberts), Department of Nutrition Chemistry, University of Nebraska, Lincoln.

Twenty-four 1-week metabolism studies which involved the weighing and analyzing of all the food eaten and the collection and analysis of all the excreta were made of young women between the ages of 16 and 25 years on self-chosen diets. The biological materials have been analyzed for calcium, phosphorus, nitrogen, iron and copper.

The average daily intakes of copper ranged from 1.4 to 3.6 mg. with an average of 2.5. The daily fecal excretions ranged from 1.2 to 2.5 mg. with a mean of 1.8. The daily urinary excretions ranged from 0.025 to 0.320 mg. with an average of 0.20. The average daily balances ranged from a retention of 1.8 mg. of copper to a loss of 0.5 mg. with an average of a positive 0.56. There were nineteen cases of storage of copper and five cases of negative balance.

There was a relation between the level of copper intake and the amount of copper stored. Whenever the copper intake was 2 mg. or more per day there was retention and whenever the intake was less than 2 mg. there was a negative balance. Indications are to the effect that a maintenance allowance of copper for girls between the ages of 16 and 25 may be 2 mg., but that if more is present in the diet it will be stored.

The effect of apple constituents on the retention by growing rats of lead contained in spray residues. J. B. Shields (by invitation), H. H. Mitchell, and W. A. Ruth (by invitation), Animal Nutriton Division, University of Illinois, Urbana.

The purpose of this experiment was to obtain data on two points: Is there a difference in the retention of lead from spray residues and from acid lead arsenate? and Does the presence of apple constituents in the diet affect the retention of lead?

The experiment was carried out as a triplicate comparison. Twelve trios of young litter mate albino rats were equated according to weight and sex. Equalized feeding was used. In each trio one rat received a diet containing 32% of dried apple powder from unsprayed apples, the second a similar diet using sprayed apples as a source of apple powder and the third a diet in which the apple powder was replaced with starch and agar. Lead arsenate was added to diets 2 and 3 to equal the lead content of 33 parts per million in the sprayed apple powder diet. The rats were fed 1000 gm. of food, sacrificed and the complete carcass was analyzed for lead.

The results showed a significantly higher storage of lead in the rats which did not receive any apple powder. There was no significant difference between the other two series of rats. The average retentions of lead were 0.329 mg. for the rat receiving sprayed apple powder, 0.346 mg. for the rat receiving unsprayed apple powder and 0.536 mg. for the rat receiving no apple powder.

A quantitative study of the limitation of fluorine toxicosis with aluminum. W. E. Anderson and J. L. Melnick (by invitation), Laboratory of Physiological Chemistry, Yale University, New Haven.

The inhibition of aluminum salts on the toxic activity of fluorine has been observed by Sharpless and later by Kempf and co-workers. From our study evidence was obtained with rats to show quantitative relations between absorption of fluorine and level of aluminum in the diet.

To a fluorine-free basal ration, 0.0313% of fluorine and 0, 1, 10 and 40 equivalents of aluminum were added. Determinations of fluorine were made in the whole body tissue and in feces and urine.

When no aluminum was added to the diet containing fluorine, the fluorine content of the body tissue was about 200 p.p.m. As the dietary aluminum was increased to 40 equivalents the above figure fell to 35 p.p.m., and at this higher level of aluminum intake, the percentage of ingested fluorine increased nearly tenfold in feces, whereas urinary fluorine decreased in a corresponding manner.

ABSTRACTS OF PAPERS READ BY TITLE

Dynamic effects of nutrients, singly and in combination. E. B. Forbes, J. W. Bratzler (by invitation), and E. J. Thacker (by invitation), Institute of Animal Nutrition, Pennsylvania State College, State College.

Two similar programs of experimentation were conducted with albino rats for the purpose of determining the dynamic effects of beef muscle protein, dextrin and lard, individually and also with the three combined.

The nutrients studied were fed in addition to a basal maintenance ration of wheat, milk powder and NaCl.

Each of the experimental programs consisted of two parallel series of tests, one on 100 gm. rats and the other on rats weighing about 240 gm. A separate group of five rats, at the standard live weight, was used for each test.

Calorimetric periods were 7 hours long, the first hour's measurements and those of any other periods during which there was considerable activity were excluded.

In seven treatments among eight the heat increments were the higher with the younger rats.

In both series of tests, and with both ages and weights of subjects, among the single nutrients the dynamic effect of the beef muscle protein was always much the highest, that of the dextrin was always intermediate and that of the lard was always much the lowest.

In every case the dynamic effect of the mixture of the three nutrients was much lower than the combined individual effects, and in three groups of tests among the four the dynamic effect of the mixture was virtually identical with that of the lard.

Production and cure of a nutritional microcytic hypochromic anemia in dogs. O. M. Helmer (by invitation), P. J. Fouts (by invitation), S. Lepkovsky, and T. H. Jukes, Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis and the Division of Poultry Husbandry, University of California, Berkeley.

Puppies and adult dogs fed a synthetic diet apparently deficient only in factor I (rat antidermatitis factor, vitamin B_6) all developed a microcytic hypochromic anemia. Loss of appetite, loss of weight and constipation accompanied the anemia. Convulsions occurred at height of anemia in three of the puppies. Addition of iron and copper to diet did not cure the anemia or prevent its increase or development. Administration by mouth of a rice polish extract containing factor I, or crystalline factor I (60 micrograms per kilo per day) was followed by a rise in reticulocytes and rapid cure of anemia, increase in appetite, gain in weight and improvement in general condition of dogs.

The specific dynamic effects of glycine, alanine and glutamic acid. M. Kriss, Institute of Animal Nutrition, Pennsylvania State College, State College.

The specific dynamic effects of glycine, alanine and glutamic acid were determined with rats when each of these amino acids was fed as a supplement to a basal maintenance ration in quantities supplying 7.5 kilogram-calories of gross energy per day, the heat production of the animals while on the maintenance ration being used as the base value. In a similar manner the specific dynamic effect of glycine was determined when it was fed in a quantity supplying 5.6 kilogram-calories per day. The dynamic effects were expressed, for comparative purposes, 1) per gram of amino acid fed, 2) per gram of amino acid deaminized, 3) per millimol of amino acid deaminized, 4) as per cent of gross energy, and 5) as per cent of metabolizable energy.

Glutamic acid exerted the greatest dynamic effect.

The dynamic effect of alanine was nearly identical with that of glycine, when expressed as per cent of metabolizable energy, but it was considerably greater than that of glycine when expressed in any other way.

The dynamic values of glycine were slightly greater at the higher level of feeding than at the lower level.

The greatest differences among the dynamic values of the different amino acids were obtained when the effects were expressed per millimol of amino acid deaminized. The smallest differences were observed among the dynamic values expressed as per cent of metabolizable energy.

The basal metabolism of the rabbit. Robert C. Lee (by invitation), Nutrition Laboratory of the Carnegie Institution of Washington, Boston, Massachusetts.

The basal metabolism was established by 196 experiments with thirty-four adult rabbits of different breeds. Their range in weight (1 to 7 kg.) enabled a study of the influence of size upon metabolism within the same species. In ascertaining the conditions required to obtain basal metabolism measurements on rabbits, the zone of thermic neutrality was found to be from 28° to 32°C., but a period of adjustment to this temperature was necessary prior to the experiment. Fasting 24 hours did not insure a respiratory quotient of fat, but the heat production remained essentially constant between the twenty-fourth and the seventy-second hour of fasting. Time of day had no influence. The standard deviation of the individual values from the average for each animal was ±6.9%. The basal heat production of rabbits cannot be expressed as a single average value, either per unit of weight or of surface area. The total heat production per 24 hours increases as a straight-line function of the weight, from 60 calories for 1-kg. rabbits to 300 calories for 7-kg. rabbits. Expressed on the basis of the two-thirds power of the body weight (surface area), the heat production increases from 600 to 816 calories per 24 hours between 1 and 7 kg., respectively.

The basal metabolism in pregnancy. G. MacLeod, C. Taylor (by invitation), E. Robb (by invitation), D. Baker (by invitation), M. O'Donahoe (by invitation), and P. McCrery (by invitation), Nutrition Laboratory, Teachers College, Columbia University, New York.

Determinations of the basal metabolism of four primipara have been made at frequent intervals during pregnancy and for varying lengths of time following delivery. In each case the basal metabolism had been determined before onset of pregnancy. Two of the four subjects were also followed through their second pregnancy. Blood pressure, vital capacity, pulse rate, respiration rate, sub-lingual temperature, sitting and standing heights and body weight (in indoor clothing without shoes) were determined routinely at the time of each determination of the basal metabolism. The Collins Benedict Roth, Sanborn Benedict and Benedict Student Respiration Apparatus have been used.

Comparisons of the basal metabolism just preceding the birth of the baby with the pre-pregnant figures show increases in calories per square meter per hour ranging from 15.6 to 23.4%, in calories per 24 hours ranging from 23.2 to 29.7%, with increases in body weight ranging from 9.1 to 25.4%.

The vital capacity, blood pressure and pulse rate show considerable fluctuating during the course of pregnancy in these four subjects. Curves will show the order of magnitude of these fluctuations and their general trend.

Physiological properties of ascorbic acid. E. W. McHenry and M. Sheppard (by invitation), School of Hygiene, University of Toronto, Toronto.

In paired feeding experiments in guinea pigs a deficiency of ascorbic acid causes a loss in weight which is accompanied by an increase in metabolic rate and a decrease in water retention. Despite the increase in metabolic rate the body fat of the deficient animals is significantly greater than in the supplemented controls. No great difference in protein or ash was observed. The changes in metabolism have been studied also in balance experiments during which animals developed ascorbic acid deficiency and were subsequently treated with the vitamin until recovery to normal was secured.

Calcium utilization in pre-school children. H. B. Pierce, R. G. Daggs, A. B. Meservey (by invitation), and W. J. Simcox (by invitation), Departments of Biochemistry and Physiology, College of Medicine, University of Vermont, Burlington.

The object of this study has been to determine the utilization of calcium and phosphorus when supplied chiefly in milk and when 200 mg. of milk calcium have been replaced with tri-calcium phosphate. Two groups of children, each comprised of two girls and three boys, served as subjects for 6 months. Five experimental periods were devised: 1) Period (6 weeks) in which children on entirely adequate diet were saturated with calcium by adding calcium lactate to basal diet, 2) period (5 weeks) in which calcium was supplied chiefly by milk, 3) period (2 weeks) in which calcium intake was reduced to approximately two-thirds that of period 2, 4) period (4 weeks) of resaturation where same amount of calcium as supplied by lactate was added in form of tri-calcium phosphate to basal diet, and 5) period (6 weeks) in which 200 mg. of milk calcium daily were replaced by equivalent amount in form of tri-calcium phosphate. With one group of children, sequence of periods was as outlined above, and with second the sequence was reversed.

At beginning of study, it was found that calcium retention was high but decreased and became fairly uniform after 3 weeks. When 200 mg. of milk calcium were replaced by the salt, the Ca and P of the latter were utilized as well, if not better than when supplied in milk.

Metabolism of food citrate by infants. A. H. Smith, L. C. Bauguess (by invitation), and D. J. Barnes (by invitation), Department of Physiological Chemistry, Wayne University College of Medicine and Harper Hospital, Detroit.

The present report deals with the metabolism of food citrates in thirteen human infants ranging in age from 3 to 14 months and maintained under carefully controlled experimental conditions. Urine and feces were collected separately and citric acid was determined on food and excreta. The experimental periods were of 3 days duration, each such period being followed by a period of 4 days when no collections were made. An adequate diet of cereal, milk, lactose and strained vegetables supplemented with ascorbic acid and vitamins A and D provided from 1415 to 3379 mg. daily of citrate, depending on the food intake. Citric acid corresponding to 4 to 20.5% of the ingested citrate was found in the excreta. In general, more citric acid appeared in the urine than in the feces, the renal excretion ranging from 3.9 to 13.3% of the daily ingested citrate in eleven of the subjects. The dietetic and metabolic significance of the observations will be discussed.

ABSTRACTS OF SYMPOSIUM PAPERS

''*The fortification of foods with vitamins and minerals*

Basic nutrition principles involved in the fortification of foods. Agnes Fay Morgan, University of California, Berkeley.

Our modern civilization has largely taken away from the food supply the qualities of wholeness and freshness which are essential for optimum nutrition. An ideal solution of the food problem thus presented would be the return of these qualities through changes in methods of processing and distributing. Failing this, artificial additions of vitamins and minerals sufficient to restore the amounts originally present might be allowed or even demanded.

The addition of excessive amounts of non-indigenous kinds of these substances to foods may be dangerous because of the possible resultant lighting up of latent deficiencies of other and perhaps unknown kinds.

The dangers of excess of vitamin D and of nicotinic acid have been plainly indicated and their use in fortifying foods should be handled with extreme caution.

The use of fortified foods and vitamin concentrates may be desirable in restricted diets and for certain diseased and unusual conditions. In digestive failures, lactation and possible pregnancy, alcoholism, diabetes, cardiac and renal disease, reduction diets and for infants fortification may be needed. To combat in the general population the 'perverse' use of vitamin-poor foods such as sugar, white flour, polished rice, alcoholic and soft drinks, fortification of these foods may be desirable until the slow process of education relegates the demand for them to the background.

Fortification of foods in a general program for better nutrition. Lydia J. Roberts, The University of Chicago, Chicago, Illinois.

Available evidence indicates that the diets of many American people are deficient in calcium, B_1 and the pellagra preventive factor, as well as in other dietary factors. The ideal method of effecting improvement would seem to be to increase the use of the protective foods and to return to some extent at least to a larger use of less highly milled cereals and other refined foods. A long educational program would be required to accomplish this end. A supplementary program of fortification might well be used as a first-aid measure, if leadership could be kept in the hands of nutrition specialists and physicians so that the public would be properly safe-guarded against exploitation. Such fortification might well include the addition of vitamin B factors to cereals, vitamin A to all butter substitutes, and similar supplementation of natural carriers. Such fortification should be without additional cost to the consumer if it is to accomplish its end, for the lower income groups are most in need. Whatever is done in fortification should be considered as only supplementary to the program of popular education toward the improvement of the diet along the lines indicated as desirable. There may still be essential dietary factors of which we are as yet unaware and we cannot trust solely to fortification but must put our major faith in a varied diet containing generous amounts of natural foods.

The public health aspects of the fortification of foods with vitamins and minerals. W. H. Sebrell, U. S. Public Health Service, Washington, D. C.

Since the purpose in fortifying foods with vitamins and minerals is to prevent the deficiency diseases, the question is discussed from the point of view of the practical value, desirability and efficiency of fortification in this respect. It is pointed out that, with the exception of vitamin D milk, which is regarded as a valuable and desirable product for infants in some areas, the fortification of foods is undesirable from the public health point of view for several reasons. The cost of fortification would tend to put these foods beyond the reach of the lowest income groups which have the highest proportion of deficient diets. It would be wasteful because the fortified products in all probability would be purchased by people not needing them. There is no method of selecting foods to be fortified and there is not sufficient information available on what vitamins or minerals are most needed or on the proper quantity to add. The desired objective can be attained more cheaply and more satisfactorily by the use of a varied diet consisting mostly of natural unrefined foods. It is also pointed out that it is better public health practice to advocate the use of a varied diet in preference to fortified foods, since our deficiency disease problem is a vague one and deficient diets usually are found to be deficient in several respects.

Governmental control problems in the fortification of foods. E. M. Nelson, Food and Drug Administration, Washington, D. C.

Practically all aspects of the desirability of fortifying foods with vitamins or minerals have long been problems of the Food and Drug Administration in considering the propriety of the representations that have been made for various vitamin and mineral preparations. Successful sales promotion of such products depends on the extent to which representations can be made that our dietaries do not supply certain food essentials in adequate amounts and also on the representations which can be made with respect to the nature of the ills or conditions which result from such deficiencies. The Food, Drug and Cosmetic Act, which was passed last year, will require more informative labeling of food products which may be fortified with vitamins or minerals. Extensive fortification of foods may possibly lead to increased and more difficult problems of control from the standpoint of assuring the consumer that the product has the vitamin content claimed. That, however, should be no deterrent if there is satisfactory evidence that an important portion of our population would benefit from such vitamin additions to staple food products. In our control work we are conscious of an extensive, rapidly growing and constantly changing industry in vitamin preparations. It is estimated that during the year 1938 more than $100,000,000 were spent for vitamin preparations by the people of the United States. Generalizations with respect to the need for fortfication of our foods frequently lead to errors. It seems desirable to consider each vitamin or mineral and each food which may be fortified, individually. Deficiencies are most likely to occur among the low-income groups, and these groups should be given prime consideration. Investigators in the field of nutrition should exert care in the wording of statements so that they do not inadvertently foster commercial practices in the exploitation of special products rather than perform a service to the general public.

Retention, restoration and fortification of vitamins in foodstuffs. Alonzo E. Taylor, General Mills, Inc., Minneapolis, Minn.

Treatment of vitamins in foodstuffs is described under retention, protective restoration, and fortification. Natural vitamins should be retained in processing whenever practicable, but this is impracticable with certain staples such as refined sugar and white flour. Whenever retention is impracticable, processors should seek restoration, either with natural or synthetic vitamin. Such restoration ought not to exceed top limit of natural occurrence. Fortification should be reserved for special instances. Restoration of vitamin B_1 and B_2 in processed grains is technically practicable, but at present prohibitively expensive. Restoration of vitamin A in milk or butter is technically practicable and not inordinately expensive. In case of vitamin D restoration in milk and butter gives too low an intake; restoration in yolk of egg is practicable but not adapted to wide usage. To make commercial milk protective without use of fish-liver oil requires fortification with vitamin D concentrate, which is practicable and not inordinately expensive. Restoration with vitamin C would not now seem necessary, since retention is successfully accomplished. Our knowledge of nicotinic acid and vitamin E is too fragmentary to admit of discussion, under headings of retention, restoration or fortification.

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